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Mari Takalo

The Effect of Genes and Diet on Alzheimer's Disease-Related Molecular Mechanisms – Influence of Ubiquilin-1 and Dietary Lipids

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ABSTRACT

Alzheimer's disease (AD) is the most common cause of dementia in the elderly and is listed amongst the top global health challenges by World Health Organization. AD is a multifactorial disease, which is affected by a complex interplay between genetic and environmental factors. The initial cause of AD is unclear, but it is increasingly evident that abnormal trafficking, processing, accumulation, and aggregation of disease-associated proteins in the brain underlie the disease. Currently, there are no disease-modifying therapies or cures available for this disease. Since the incidence of AD is expected to exponentially increase, due to aging of the population, there is an urgent need to find ways to reduce the risk, identify novel biomarkers, and harness potential therapeutic targets. For these purposes, a better understanding of the underlying molecular mechanisms of AD pathogenesis is required. The aim of this thesis was to characterize the effect of genes and dietary manipulation on AD-related proteins and pathways, in particular those related to β-amyloid (Aβ), tau, and neuroinflammation. The special emphasis was on the AD-associated risk gene *UBQLN1* and on the influence of dietary lipid composition.

The goal in study I was to review the role of ubiquilin-1 in AD pathogenesis and as a potential therapeutic target in AD on the basis of recent literature. In study II, the aim was to experimentally characterize the expression and function of ubiquilin-1 in human brain as well as in both, *in vitro* and *in vivo* models of AD. Ubiquilin-1 has previously been shown to regulate the levels, processing, trafficking, degradation and accumulation of several AD-associated proteins. Study II provides evidence for a novel function for ubiquilin-1 as β-site APP cleaving enzyme-1 (BACE1)–interacting protein. This interrelationship was found to increase BACE1 stability by diverting it from the lysosomal degradation pathway and this may subsequently affect Aβ accumulation. Although ubiquilin-1 has previously been shown to alleviate the effects of different types of stress, it was not found to have a similar role during AD-associated neuroinflammation in mouse brain or in cell-based models. Together with previous reports, these data indicate that ubiquilin-1 has an evident, but complex role in AD molecular pathogenesis. Based on its several AD-associated functions, ubiquilin-1 might be a plausible therapeutic target. However, a deeper understanding of its multifaceted role and functions is necessary.

Studies III and IV investigated the effects of dietary manipulation on AD-related molecular mechanisms and cognitive changes in aging mice. In study III, the four month intervention with the high-fat diet (HFD) was found to increase tau expression, and tau exon 10 inclusion, independently of AD or type 2 diabetes-related genetic background. HFD-induced changes in tau expression were correlated with a poorer performance in cognitive tests, but were not associated with central or peripheral insulin signaling, neuroinflammation or the expression of tau exon 10 splicing factors. These findings suggest that HFD may accelerate tau-related pathology and associate with the risk or progression of AD and other neurodegenerative tauopathies.

In study IV, nine-month intervention with special lipid-based diets containing fish oil (FO), fish oil and plant sterols (FOPS) or a special multinutrient combination including precursors and cofactors for neuronal membrane formation (Fortasyn) were found to alleviate the odor recognition impaiment of the AD mice. In contrast, only Fortasyn diet alleviated the spatial learning deficits of the AD mice. All diets markedly inhibited the APP-cleaving enzymes, β- and γ-secretase, in the brain of AD mice, but this was translated to reduce Aβ levels only in mice fed with FOPS-diet. The alleviated cognitive impairment by the dietary intervention was neither related to changes in neuroinflammation nor oxidative stress. Therefore, this implies that additional supplementation with specific nutrients may improve the cognitive benefits of fish oil-containing diets through Aβ-independent mechanisms. Together, the data from studies III and IV indicate that modulation of dietary lipid and nutrient composition may be a potential approach for delaying the progression of AD-related pathological changes and the subsequent cognitive decline.

In summary, the results obtained from the studies in this thesis provide novel information about the functional role of specific genetic and environmental factors associated with AD pathogenesis. These findings may be applied to the identification of novel pharmacological targets, predictive and/or diagnostic biomarkers as well as the development of refined strategies to intervene with disease progression, in order to advance general well-being in the aging societies and help the management of neurodegenerative disorders.

National Library of Medicine Classification: WT 155, QU 470, QU 477, QT 235, QU 86
Medical Subject Headings: Alzheimer Disease; Protein Aggregation, Pathological; Genes; Diet; Diet, High-Fat; Dietary Fats; Lipids; Fish Oils; Food, Formulated; Cognition; Disease Models, Animal; Mice

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TIIVISTELMÄ

Alzheimerin tauti (AT) on yleisin dementiaa aiheuttava sairaus ikääntyvillä. AT:iin ei ole olemassa hoitoa, jolla voitaisiin estää taudin puhkeaminen tai hidastaa sen etenemistä. Myöhäisiällä puhkeava AT on monitekijäinen sairaus, jonka syntyyn tiedetään vaikuttavan joukko geneettisiä sekä ympäristöön ja elämäntapoihin liittyviä riskitekijöitä. Näiden riskitekijöiden yhteisvaikutuksesta alttius patologisille solutason muutoksille lisääntyy ikääntymisen myötä ja AT:iin liittyvien proteiinien, kuten taun ja amyloidiprekursoriproteiinista (APP) pilkottavan β-amyloidin (Aβ), epänormaali prosessointi, hajotus ja kertyminen yleistyvät. Proteiinien epänormaali kertyminen sekä siihen liittyvät stressitilat puolestaan ovat vahingollisia hermosoluille ja johtavat lopulta laajamittaiseen aivokudoksen tuhoutumiseen. Tästä huolimatta AT:n perimmäinen syy sekä siihen liittyvät biologiset mekanismit ovat edelleen suurelta osin heikosti tunnettuja. AT:n riskitekijöiden tunnistaminen ja taudin taustalla vaikuttavien solu- ja molekyylitason mekanismien yksityiskohtainen selvittäminen on ehdottoman tärkeää taudin ennaltaehkäisemiseksi ja uusien terapiamuotojen kehittämiseksi. Tässä väitöskirjatutkimuksessa tavoitteena oli kartoittaa tiettyjen riskigeenien ja ruokavalion ravintoaineiden vaikutusta AT:in liittyviin molekyylitason mekanismeihin AT-potilaiden aivokudosnäytteissä sekä erilaisissa solu- ja eläinmalleissa sekä kognitiivisiin muutoksiin hiirillä.

Kahdessa ensimmäisessä osatyössä selvitettiin AT:n riskigeenin, ubikiliini-1:n, kytkeytymistä AT:n patologiaan. Aikaisemman kirjallisuuden perusteella ubikiliini-1 säätelee aivoissa useiden AT:iin liittyvien proteiinien tasoja sekä hajotusta ja voi siten liittyä AT:n varhaisiin solutason muutoksiin. Toisessa osatyössä AT-potilaiden aivokudosnäytesarjasta sekä solu- ja eläinmallipohjaisista kokeista saadut tulokset osoittivat uuden yhteyden ubikiliini-1:n ja APP:a pilkkovan β-sekretaasi-entsyymin (BACE1) välillä. Tässä tutkimuksessa ubikiliini-1:n havaittiin lisäävän BACE1:n tasoja soluissa estämällä sen kulkeutumisen soluissa lysosomaaliseen hajotukseen. Tämä puolestaan voi johtaa taudissa sakkautuvan Aβ:n lisääntyneeseen tuotantoon sekä kertymiseen ja siten taudin patologian etenemiseen. Vaikka aikaisemmissa tutkimuksissa ubikiliini-1:n on todettu suojaavan soluja erilaisissa stressitiloissa, ei vastaavanlaista yhteyttä havaittu tässä tutkimuksessa ubikiliini-1:n ja AT:iin keskeisesti liittyvän neuroinflammaation välillä. Näiden tulosten sekä aikaisempien löydösten perusteella ubikiliini-1:llä vaikuttaa olevan tärkeä, mutta monitahoinen merkitys AT:n patogeneesissä.

Kolmannessa ja neljännessä osatyössä oli pyrkimyksenä selvittää ruokavalion rasvahappojen ja eräiden muiden ravintoaineiden vaikutusta AT:n patogeneesiin sekä kognitiivisiin muutoksiin hiirimalleilla. Aikaisemmissa tutkimuksissa on näytetty, että runsaasti tyydyttyneitä rasvoja sisältävä ruokavalio, ylipaino ja tyyppi 2:n diabetes (T2D) lisäävät AT:n riskiä. Kolmannessa osatyössä runsaasti tyydyttyneitä rasvahappoja sisältävän rasvadieetin todettiin merkittävästi lisäävän AT:ssa keskeisen tau-proteiinin tasoja, mRNA:n ilmentymistä sekä vaihtoehtoista silmukointia, jonka seurauksena tau-proteiinien isoformien suhteellinen määrä muuttui hiirimallin aivoissa. Nämä

rasvadieetistä johtuvat tau-muutokset liittyivät heikentyneeseen suoriutumiseen muistia ja kognitiivista suoriutumista mittaavissa käyttäytymiskokeissa. Muutokset olivat osittain yhteydessä rasvadieetillä olleilla hiirillä havaittuun painon nousuun, mutta suurelta osin riippumattomia T2D:een liittyvistä glukoosi- ja insuliinisignaloinnin häiriöistä tai aivojen neuroinflammaatiosta. Tulosten perusteella runsaasti tyydyttyneitä rasvoja sisältävällä ruokavaliolla voi olla yhteys tau-patologiaan sekä heikentyneeseen muistiin ja oppimiseen ja se voi näin ollen altistaa AT:lle ja muille hermorappeumasairauksille.

Ravinnosta saatavan kalaöljyn on aikaisemmin osoitettu pienentävän AT:n riskiä. Neljännessä osatyössä erilaisten kalaöljypohjaisten rasvadieettien (kalaöljy, kalaöljy + kasvisterolit tai Fortasyn) havaittiin merkittävästi parantavan AT:n siirtogeenisten hiirten heikentynyttä suoriutumista hajumuistia ja -tunnistusta mittaavassa testissä. Lisäksi Fortasyn-ravintolisän, joka sisältää mm. erilaisia solukalvojen rakennusaineiksi tarvittavia lipidejä ja vitamiineja, havaittiin tehostavan kalaöljypohjaisen ruokavalion hyödyllistä vaikutusta AT-hiirten sijaintimuistiin. Vaikka jokaisen kalaöljypohjaisen dieetin havaittiin tehokkaasti vähentävän APP:a pilkkovien β- ja γ-sekretaasientsyymien aktiivisuutta, ei tämä heijastunut vähentyneeseen Aβ:n määrään. Nämä tulokset tukevat ravinnon kalaöljyn hyödyllisiä vaikutuksia AT:iin liittyviin molekyylitason ja kognitiivisiin muutoksiin. Lisäksi tutkimus osoittaa, että erityiset lisäravinteet saattavat tehostaa kalaöljyn positiivisia vaikutuksia Aβ-patologiasta riippumattomin mekanismein. Kaiken kaikkiaan kolmas ja neljäs osatyö osoittavat, että ravinnon rasvasisällöllä voi olla merkitystä AT:n riskin, etenemisen ja oireiden kannalta.

Tämä väitöskirjatyö valaisee tutkittujen geneettisten ja ravintoperäisten tekijöiden merkitystä AT:n patogeneesissä sekä kognitiivisissa muutoksissa. Tätä uutta tietoa voidaan mahdollisesti tulevaisuudessa hyödyntää AT:a ennakoivien tai diagnostisten biologisten merkkiaineiden sekä uusien terapiamuotojen kehittämisessä. Siten nämä tutkimustulokset voivat edistää hyvinvointia ikääntyvässä yhteiskunnassa sekä auttaa muistisairauksien hoidossa ja ennaltaehkäisyssä.

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Mari Takalo

List of Original Publications,

This dissertation is based on the following original publications:

I **Takalo M**, Haapasalo A, Natunen T, Viswanathan J, Kurkinen KMA, Tanzi RE, Soininen H, Hiltunen M. Targeting ubiquilin-1 in Alzheimer's disease. *Expert Opinion on Therapeutic Targets 17:795-810, 2013*

II Natunen T[#], **Takalo M**[#], Kemppainen S, Leskelä S, Marttinen M, Kurkinen KMA, Pursiheimo JP, Sarajärvi T, Viswanathan J, Gabbouj S, Solje E, Tahvanainen E, Pirttimäki T, Kurki M, Paananen J, Rauramaa T, Miettinen P, Mäkinen P, Leinonen V, Soininen H, Airenne K, Tanzi RE, Tanila H, Haapasalo A[*], Hiltunen M[*]. Relationship between ubiquilin-1 and BACE1 in human Alzheimer's disease and APdE9 transgenic mouse brain and cell-based models. *Article in press in Neurobiology of Disease. [#*] These authors contributed equally to this study.*

III **Takalo M**, Haapasalo A, Martiskainen H, Kurkinen KMA, Koivisto H, Miettinen P, Khandelwal VKM, Kemppainen S, Kaminska D, Mäkinen P, Leinonen V, Pihlajamäki J, Soininen H, Laakso M, Tanila H, Hiltunen M. High-fat diet increases tau expression in the brain of T2DM and AD mice independently of peripheral metabolic status. *Journal of Nutritional Biochemistry 25:634-641, 2014*

IV Koivisto H, Grimm M, Rothhaar T, Berkecz R, Lütjohan D, Giniatullina R, **Takalo M**, Miettinen P, Lahtinen HM, Giniatullin R, Penke B, Janáky T, Broersen L, Hartmann T, Tanila H. Special lipid-based diets alleviate cognitive deficits in the APPswe/PS1dE9 transgenic mouse model of Alzheimer's disease independent of brain amyloid deposition. *Journal of Nutritional Biochemistry 25:157-169, 2014*

Contents

Abbreviations

aa	Amino acid
AD	Alzheimer's disease
ADAM	A disintegrin and metalloprotease family
ALP	Autophagosome-lysosome pathway
ALS	Amyotrophic lateral sclerosis
APdE9	Transgenic mouse line bearing human *APP* with the "Swedish" mutations and human *PSEN1* with a deletion of exon 9
Aph1	Anterior pharynx-defective 1
ApoE	Apolipoprotein E
APP	Amyloid precursor protein
ARF6	ADP ribosylation factor 6
Asp-Pro-rich repeats	Asparagine- and proline-rich repeats
Aβ	β-amyloid peptide
BACE1	β-site APP cleaving enzyme 1
BBB	Blood brain barrier
BSA	Bovine serum albumin
CAA	Cerebral amyloid angiopathy
CNS	Central nervous system
CSF	Cerebrospinal fluid
DLBD	Diffuse Lewy body disease
DMEM	Dulbecco's modified eagles medium
EGFP-F	Enhanced farnesylated green fluorescent protein
EH	Epidermal growth factor substrate 15 homology domain
eIF2α	Eukaryotic translation initiation factor 2α
EOAD	Early onset Alzheimer's disease
ER	Endoplasmic reticulum
FAD	Familial Alzheimer's disease
FBS	Fetal bovine serum
FO	Fish oil
FOPS	Fish oil and Plant sterol
FTD	Frontotemporal degeneration
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
GFAP	Glial fibrillary acidic protein
GFP	Green fluorescent protein
GGA	Golgi-localized γ-ear containing ADP ribosylation-binding factor
GlcNAc	N-acetyl-D-glucosamine
GTT	Glucose tolerance test
GWAS	Genome-wide association studies
HFD	High-fat diet
HMW	High molecular weight
IFN-γ	Interferon gamma
IGF2	Insulin-like growth factor 2
IL1β	Interleukin 1β
i.p.	Intra-peritoneal
ITT	Insulin tolerance test
LEL	Late endosomes/lysosomes
LPC	Lysophosphatidylcholine
LPS	Lipopolysaccharide
MAPT	Tau gene
MAP	Microtubule associated protein
MCI	Mild cognitive impairment
mRFP	Monomeric red fluorescent protein
MT	Microtubule
mTOR	Protein kinase mammalian target of rapamycin
MUFA	Monounsaturated fatty acid
Nct	Nicastrin
NFT	Neurofibrillary tangle
NINCDS-ADRDA	National Institute of Neurological and Communicative Disorders and Stroke and the Alzheimer's Disease and Related Disorders Association
PBS	Phosphate-buffered saline
PC	Phosphatidylcholine
PD	Parkinson's disease
PDL	Poly-D-lysine
PE	Phosphatidylethanolamine
PEN2	Presenilin enhancer-2
PET	Positron emission tomography
PFA	Paraformaldehyde
PHF	Paired helical filament

PLIC-1	Protein linking integrin-associated protein to cytoskeleton-1, ubiquilin-1
PM	Plasma membrane
PQC	Protein quality control
PS1, -2	Presenilin-1 and -2
PSEN1, -2	Presenilin-1 and -2 encoding genes
PUFA	Polyunsaturated fatty acid
ROS	Reactive oxygen species
s.c.	Sub-cutaneous
SF	Straight filament
SFA	Saturated fatty acid
SH3	SRC homology 3 domain
SNP	Single nucleotide polymorphism
SQSTM1/ p62	Sequestosome-1
Srsf	Serine/arginine-rich splicing factors
STD	Standard diet
TNFα	Tumor necrosis factor-α
TPER	Tissue protein extraction buffer
TV	Transcript variant
TWD	Typical Western-type diet
T2DM	Type-2 diabetes mellitus
UBA	Ubiquitin-associated domain
UBL	Ubiquitin-like domain
UBQLN1	Ubiquilin-1 gene
UBQLN2	Ubiquilin-2 gene
UMP	Uridine monophosphate
UPR	Unfolded protein response
UPS	Ubiquitin-proteasome system
WHO	World Health Organization
4R-tau	4-repeat tau protein isoforms
3R-tau	3-repeat tau protein isoforms

1 Introduction

Age-related dementia is listed as one of the high priority global public health challenges by World Health Organization (WHO). It is estimated that almost 50 million people worldwide suffer from dementia and the number is predicted to triple by the year 2050 (World Alzheimer Report 2014). The vast majority of dementia is defined as Alzheimer's disease (AD) (Qiu et al., 2009). AD is characterised by a gradual decline in both memory and cognitive functions, which significantly interfere with basic daily life (Morris et al., 1993). AD is one of the leading causes of disability and the sixth highest cause of death in those of 65 years of age and older (Qiu et al., 2009). The prevalence of AD increases exponentially with age and approximately a third of individuals older than 85 years have some signs of dementia (Mayeux and Stern, 2012, Qiu et al., 2009). More than 70% of these are caused by AD. Without either a cure or disease-modifying therapies, the number of affected individuals will drastically increase during the following decades (Kumar et al., 2015). Together with prolonged life expectancies, and the resultant increase in the average age of the population, this will cause enormous global social, economic and medical challenges. It has been suggested that a therapy providing even a modest delay in the onset of AD, would yield hundreds of billions of savings in global medical care costs (Changing the Trajectory of Alzheimer's Disease, a report by Alzheimer's association). However, the currently approved drugs for the treatment of AD patients provide only relatively short-term symptomatic improvements and have no effect on the underlying pathogenic mechanisms or progression of the disease. For successful clinical drug trials, it will be necessary to improve methods to accurately and efficiently identify individuals at increased risk for AD already at the early stages of disease and find biomarkers that correlate well with the clinical onset and progression of AD.

At the biological level, AD is a progressive neurodegenerative disease, which leads to massive atrophy of affected brain areas. It is increasingly evident that abnormal processing, accumulation, and aggregation of specific proteins or protein fragments in the brain underlies most neurodegenerative diseases, including AD (Ross and Poirier, 2004). Two pathological hallmark aggregates found in AD brain are extracellular amyloid plaques and intraneuronal neurofibrillary tangles (NFTs). Amyloid plaques are formed as a result of accumulation of β-amyloid peptides (Aβ) that aggregate together, whereas NFTs consist of filaments of abnormally phosphorylated tau protein. Other central players in AD molecular pathogenesis are the β-secretase, namely BACE1 (β-site APP cleaving enzyme 1), and the γ-secretase. These two are the key enzymes responsible for Aβ peptide production. The amyloid cascade hypothesis postulates, that Aβ deposition is an early and initial event in AD. Via direct and indirect mechanisms, it triggers a cascade of other pathological incidences, which eventually together lead to neuronal cell death and onset of clinical disease. How tau aggregation contributes to AD onset, is less understood. However, tau deposition is sufficient to cause several other brain disorders, most of which are Aβ-independent, suggesting that tau is more than just a byproduct of Aβ accumulation in AD. Although these neuropathological changes in AD brain are relatively well characterized, the primary cause of Aβ accumulation, NFT formation, synaptic loss and neurodegeneration still remains partly unresolved. An understanding of these fundamental mechanisms and the molecular players

involved is therefore urgently needed, in order to discover new potential diagnostic and therapeutic targets.

The major risk factor for AD is advanced age. Normally AD is diagnosed at the age of ~65 years at the earliest. However, approximately 5% of all AD cases occur earlier, even decades before the age of 65. About 10% of these early onset AD (EOAD) cases are familially inherited AD (FAD) and are caused by autosomal dominant mutations in three genes, that encode the amyloid precursor protein (APP), presenilin-1 (PS1), and presenilin-2 (PS2) proteins, individually. All mutations in *APP*, *PSEN1,* and *PSEN2* lead to increases in the levels or accumulation of Aβ and thus support the amyloid cascade hypothesis (Borchelt et al., 1996, Citron et al., 1992, Gessel et al., 2012, Murakami et al., 2002, Potter et al., 2013). On the other hand, no clear genetic association between increased Aβ production and the more common late onset form of AD (LOAD) have been found. LOAD is a heterogeneous disease, which is associated with a complex interplay between common genetic risk variants and environmental factors. The most important risk factors of LOAD after age are positive family history of dementia, ε4 allele of the apolipoprotein E encoding gene (*APOE ε*4), and female gender. In addition, combined data from epidemiologic and experimental studies suggest that co-morbidities and metabolic factors, such as hypertension, dyslipidemia, overweight and obesity, type 2 diabetes mellitus (T2DM), vascular disorders, depression, brain traumas, and stress increase the risk of AD (Sindi et al., 2015). Importantly, most of these risk factors are modifiable through certain drugs and lifestyle choices, such as smoking, alcohol consumption and dietary manipulation. Since there is neither cure nor disease-modifying therapies available for AD, it is of great importance to recognize and harness all plausible ways to intervene with disease development or progression.

The aim of this thesis was to characterize the functional mechanisms of specific genes and dietary manipulation on AD-related proteins and pathways, such as BACE1, γ-secretase, APP, Aβ and tau, and neuroinflammation. Special emphasis was on the AD-associated gene *UBQLN1*, encoding the ubiquilin-1 protein, and possible mechanisms of its action in this disease. In previous studies, ubiquilin-1 has been shown to be an important regulator of cellular functions, such as protein trafficking, degradation and aggregation, which are linked to mechanisms of AD and neurodegeneration (Bertram et al., 2005, El Ayadi et al., 2012, Haapasalo et al., 2010, Hiltunen et al., 2006, Lu et al., 2009, Mah et al., 2000, Stieren et al., 2011). In addition, the influence of dietary fatty acid composition on AD-related molecular and behavioral changes in mice was investigated in detail. The results obtained from these studies provide novel information regarding the underlying molecular mechanisms of AD in experimental models, which can be applied in order to recognize effective prevention strategies, identify novel biomarkers, and find new pharmacological therapeutic targets for AD.

2 *Review of the literature*

2.1 CLINICAL AND NEUROPATHOLOGICAL FEATURES OF ALZHEIMER'S DISEASE

AD is distinguished from normal age-related cognitive changes with a slow but inevitably progressing decline, which is severe enough to cause social and occupational incapacity and interfere with daily life (criteria by The National Institute of Neurological and Communicative Disorders and Stroke and the Alzheimer's Disease and Related Disorders Association, NINCDS/ADRDA and The Diagnostic and Statistical Manual of Mental Disorders, Fourth Edition, DSM-IV) (McKhann et al., 1984, McKhann, 1994). AD onset generally takes place around 65 years of age at the earliest, although in some rare cases, AD can be diagnosed decades earlier. Usually, the first symptoms include difficulties to recall, and take advantage of, recently learned information. Problems to perform complex tasks, such as organizing, planning, problem solving and reasoning, are also common. Patients often have difficulties in recognition, time management, and spatial orientation, with changes in mood and personality also being typical. Gradually, the disease progresses to affect all intellectual functions and even the most basic daily functions become challenging. Loss of speech and ability to move independently are often associated with the most advanced stages. Eventually, AD leads to premature death, on average, eight to ten years after the initial clinical diagnosis (Tarawneh and Holtzman, 2012).

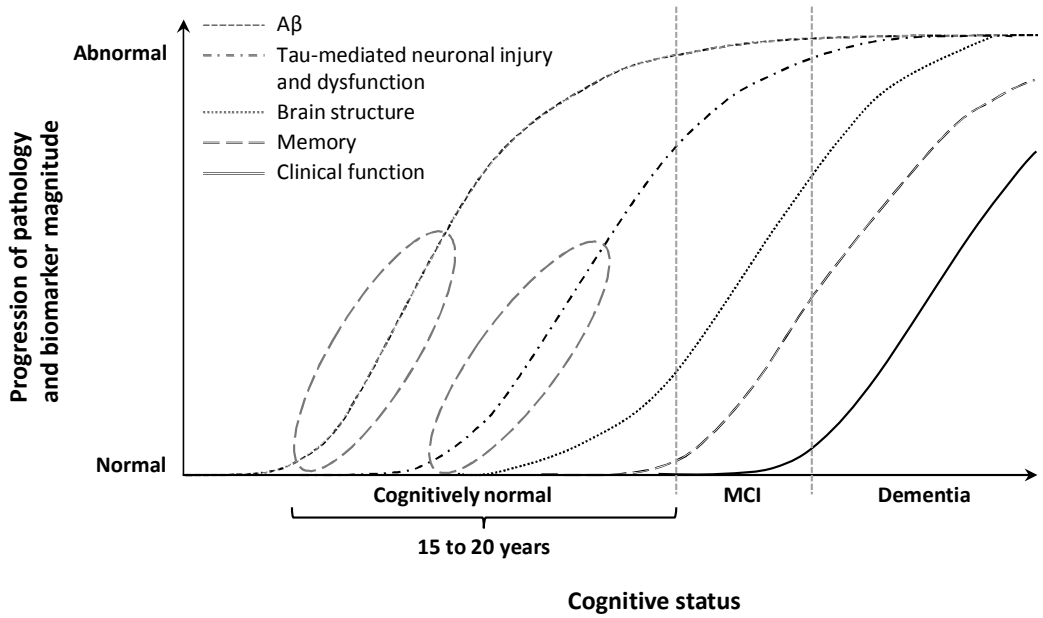

Figure 1. Progression of neuropathological events during the course of Alzheimer's disease (AD). Aβ accumulation in the brain precedes mild cognitive impairment (MCI) and clinical dementia at least by 15 years and progresses only gradually after the symptoms have occurred. This can be detected as reduced Aβ levels in the cerebrospinal fluid (CSF). Progression of neurofibrillary pathology and neuronal injury correlate better with the clinical symproms. However, increased total and phosphorylated tau can also be detected in the CSF already 10-15 years before the onset of clinical symptoms. The brain structure may be already severely and irreversibly damaged when the first clinical signs occur and the diagnosis is made. The optimal time for starting a therapy against Aβ and tau accumulation (indicated with dashed ellipses) would therefore precede the onset of clinical dementia (indicated with vertical dashed lines) by years. This would require early, efficient and accurate identification individuals destined to progress towards AD. (Adapted from Jack, et al., 2010, The Lancet Neurology 9:119-128, with the kind permission of Elsevier).

A positive diagnosis of AD is usually made when the first symptoms become so evident that the patient seeks clinical examination. This raises major concerns, because at the time when the cognitive changes start to appear, the brain pathology is already well developed and likely to be irreversible. In order to successfully prevent these irreversible changes, it would be necessary to start treating the patients at the time when the brain pathology is not yet fully developed and potentially modifiable, preferably even 15 to 20 years prior to the cognitive dysfunction (**Figure 1**)(Jack et al., 2010). However, due to difficulties to accurately and efficiently identify individuals at risk, it has been challenging to develop disease-modifying therapies for AD. Thus, currently all approved AD drugs [N-methyl-D-aspartate (NMDA) receptor antagonists and cholinesterase inhibitors] provide only symptomatic improvements and have no effect on the underlying pathogenic mechanisms or progression of the disease. Moreover, all disease-modifying therapies that have so far reached clinical trials have consistently failed, if not because of dose-limiting side-effects, then mostly due to the fact that the treatment has been started too late regarding the progression of pathology

(Coric et al., 2012, Cummings et al., 2013, Doody et al., 2013, Haapasalo and Kovacs, 2010, Salloway et al., 2009, Sugino et al., 2015).

Increasing amount of data suggest that biomarkers in combination with detailed neuropsychiatric assessment can significantly increase the specificity and accuracy of AD diagnosis (Cummings et al., 2013, Jack et al., 2011). Nowadays, cerebrospinal fluid (CSF) biomarker levels (Aβ, total tau, phospho-tau), structural and/or functional brain imaging [magnetic resonance imaging (MRI) and positron emission tomography (PET)], family history as well as genetic information are increasingly applied to yield earlier and more specific and sensitive diagnosis (McKhann et al., 2011). The first appearing sign of evolving AD pathology is Aβ accumulation, which is followed by tau-related changes and brain atrophy (**Figure 1**) (Bateman et al., 2012, Fagan et al., 2014, Jack et al., 2010, Klunk et al., 2004, Sunderland et al., 2003). Although progression of both Aβ- and tau-related pathologies in the brain can be estimated based on their levels in the CSF (Blennow et al., 2012, Sunderland et al., 2003), the final definition of the diagnosis is still based on neuropathological findings in detailed *post mortem* histological examination. Two characteristic neuropathological lesions found in AD brain are the β-amyloid plaques formed of Aβ peptides in the brain parenchyma and the intraneuronal NFTs, which are composed of hyperphosphorylated and misfolded tau protein (Goedert et al., 1991). These features are accompanied by increased activation of brain inflammatory cells, astrocytes and microglia (Heneka et al., 2015). In addition, massive neuronal cell death, which is preceded by oxidative stress and neuroinflammation, impaitred neurotransmission, synaptic dysfunction and loss, axonal degeneration and dystrophy of dendritic trees, is centrally involved in this disease's pathogenesis (Crews and Masliah, 2010, Nimmrich and Ebert, 2010). At the macroscopic level in the brain, the neuronal loss is observed as symmetric cortical atrophy of the hippocampus and the medial temporal lobes with an accompanying dilatation of the lateral ventricles (Scheltens et al., 1995).

2.1.1 β-Amyloid Plaques

Synaptic and neuronal loss in AD is believed to result from the abnormal processing and deposition of specific proteins into the lesions described above. The β-amyloid plaques are formed from Aβ peptides, which are themselves produced from APP through sequential proteolytic cleavages conducted by the β- and γ-secretase enzymes (Glenner and Wong, 2012, Kang et al., 1987, Masters et al., 1985, Selkoe, 1994). Soluble Aβ monomers have a strong tendency to self-aggregate and accumulate as oligomers that can readily stick together to form insoluble fibrils. Eventually they form amyloid plaques in the brain parenchyma or cerebral blood vessel walls (cerebral amyloid angiopathy, CAA)(Masters et al., 1985). During AD, Aβ accumulates mainly in the isocortex, while allocortex, brain stem, and cerebellum are affected to a lesser extent. A considerable number of amyloid plaques are commonly found in the brain of elderly individuals with good cognitive health. However, these so called diffuse amyloid plaques, are morphologically different from the dense core amyloid plaques, typically detected in the *post mortem* brain of AD sufferers. In contrast to the diffuse plaques, dense-core plaques contain Aβ peptides orientated in a β-pleated sheet conformation and are associated with neurotoxic effects, such as presence of abnormal neuronal processes (dystrophic neurites), activation of astrocytes and microglia, and degeneration of nearby synapses and neurons. The distribution of amyloid plaques in AD brain does not seem to

follow any well predictable regional pattern, correlate with neurodegeneration, or associate with the progression and duration of the disease (Ingelsson et al., 2004). This, however, is likely because Aβ-related changes mostly take place at the very early stages of the disease. Thus, Aβ accumulation reaches or has already reached a saturation plateau at the time clinical signs of AD begin to clearly manifest themselves (**Figure 1**) (Ingelsson et al., 2004, Serrano-Pozo et al., 2011). In addition, some evidence implies that soluble Aβ oligomers, rather than fibrils or plaques, are responsible for the toxic effects and correlate better with the degree of synaptic loss and cognitive decline (Findeis, 2007, Mucke et al., 2000, Selkoe, 2008).

2.1.2 Neurofibrillary tangles

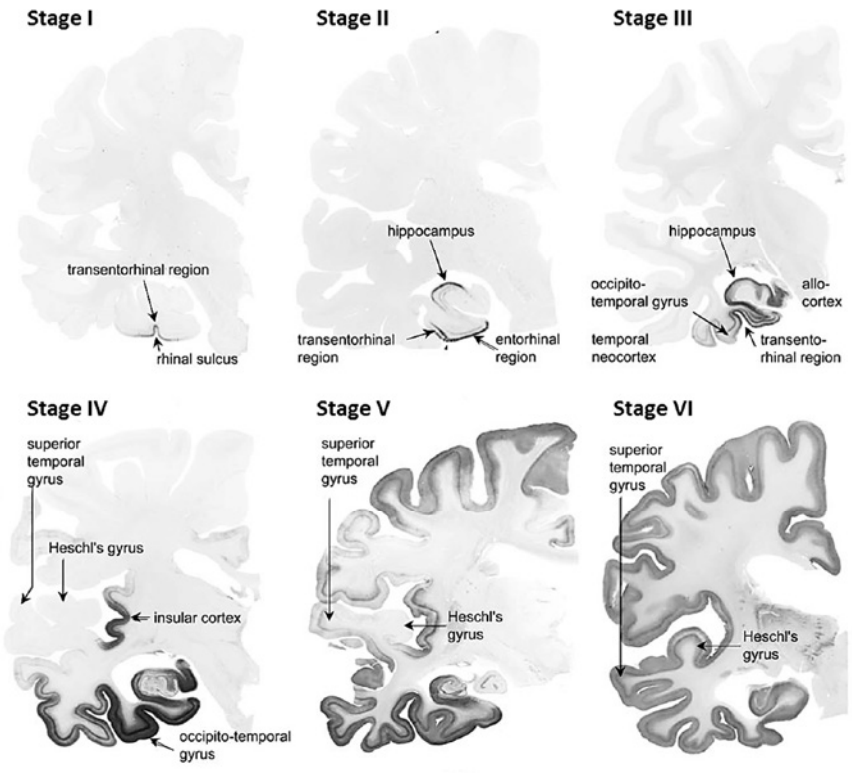

Figure 2. Progression of neurofibrillary pathology at the different Braak stages. NFTs are detected from *post mortem* brain slices with AT8 antibody, which detects tau phosphorylated at amino acid positions Ser199, Ser202 and Thr205. At transentorhinal stage (Braak stages I and II), the pathology initiates at the transentorhinal and entorhinal regions and progresses to the hippocampus. These stages are usually asymptomatic. At the limbic stage (Braak stages III and IV), the hippocampal pathology advances and NFTs spread to the medial temporal lobe. At these stages, the first signs of MCI or early AD usually occur. At the neocortical stage (Braak stages V and VI), the neurofibrillary pathology covers the whole isocortex and the patients usually have fully developed AD. (Adapted from Braak et al., 2006, Acta Neuropathol 112:389-404, with kind permission of Springer Science and Business Media).

NFTs, the other hallmark aggregates of AD brain, are composed of hyperphosphorylated tau protein, which is misfolded in the form of paired helical filaments (PHFs), straight filaments (SF), and twisted ribbon-like assemblies (Grundke-Iqbal et al., 1986). NFTs in the brain can be detected in three modalities that are thought correlate to different stages: diffuse pre-tangles existing in normal-appearing neurons, mature intraneuronal tangles occuring in dystrophic neurons, and extracellular "ghost" tangles left behind after neuronal death (Augustinack et al., 2002, Braak et al., 1994, Su et al., 1993). In contrast to the β-amyloid plaques, the spreading of NFTs in the brain is highly hierarchical and predictable. Progression of the neurofibrillary pathology is well correlated with neurodegeneration, as well as with the severity, neuropsychiatric profile and duration of the disease. Six stages of AD can be classified on the basis of NFT distribution and this Braak staging has been used as part of diagnostic criteria since 1997 (**Figure 2**)(NI-RI consensus 1997) (Braak and Braak, 1995). At the transentorhinal stage (Braak stages I-II), neurofibrillary pathology can be detected at the transentorhinal and entorhinal cortex and individuals are usually clinically asymptomatic or may suffer from mild cognitive defects. At the limbic stage (Braak stages III-IV), the pathology affects the whole hippocampus and extends to neocortical association areas. These individuals are usually detected with a mild to moderate clinical disease phenotype. At the neocortical stage (Braak stages V-VI), the pathology covers the whole associative neocortex and patients are usually diagnosed with severe AD (**Figure 2**). The Braak staging is based on *post mortem* immunostaining of phosphorylated tau with the AT8 antibody, which detects AD-specific phosphorylation of tau at amino acid positions serine 199 and 202, and Threonine 205 (see also **Chapter 2.2.3.2**) (Braak et al., 2006). Despite the clear correlation between tau accumulation and disease progression, the pathogenic mechanisms of tau in AD are not that well established. It is still under debate whether tau-related changes have a causative role or whether they occur as a byproduct of other pathological events in AD, such as Aβ accumulation. However, it is worth mentioning, that tau hyperphosphorylation and accumulation are not specific for AD, but is associated several brain disorders, most of which are Aβ-independent (Spillantini and Goedert, 2013). Furthermore, although mutations in the tau encoding gene (*MAPT*) are not associated with AD, some specific ones are known to be sufficient to cause other neurodegenerative diseases, such as a familial form of frontotemporal degeneration (FTD). Together these data argue for an underlying role of tau deposition in AD, and beyond.

2.1.3 The amyloid cascade hypothesis

The most prominent theory elucidating the occurrence of molecular events in AD, the so called 'amyloid cascade hypothesis', postulates that Aβ accumulation is the primary initiating event in AD (Hardy and Higgins, 1992). According to the hypothesis, Aβ deposition triggers secondary pathological events, including oxidative stress, neuroinflammation, tau hyperphosphorylation and the subsequent NFT formation (**Figure 3**). As a consequence, these changes, together lead to synaptic and neuritic injury, neuronal loss and the onset of dementia. Although detailed molecular mechanisms of AD still partially remain a mystery, the amyloid cascade hypothesis is strongly supported by AD genetics, clearly demonstrating that mutations inducing lifelong increase in Aβ accumulation are

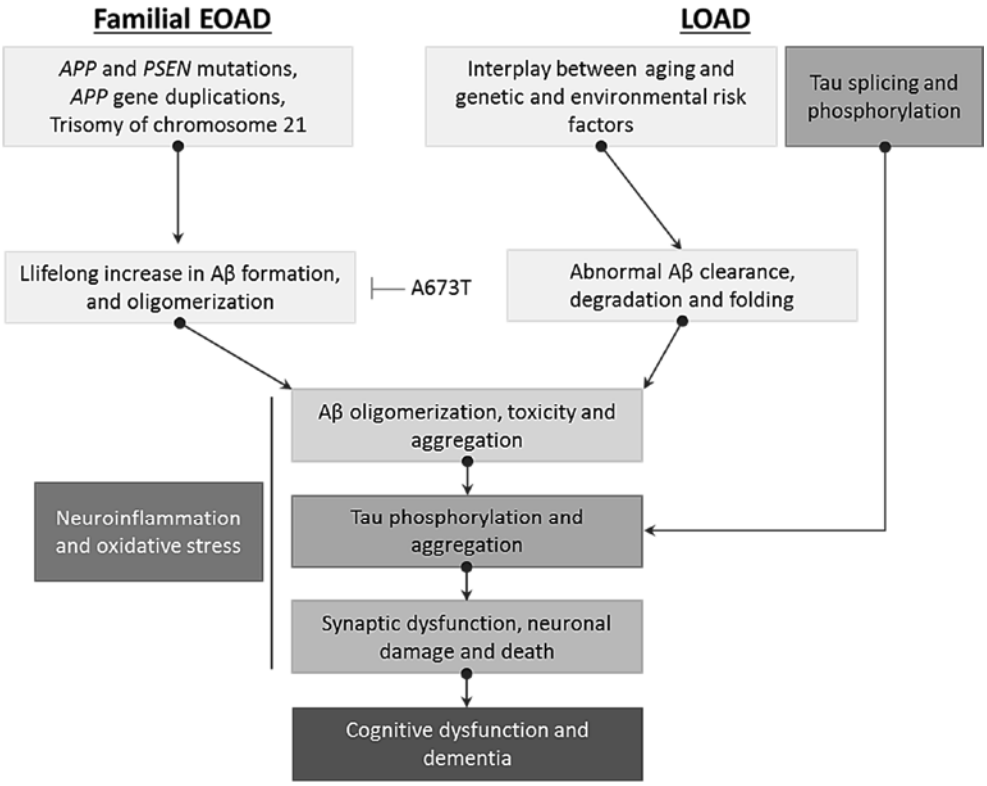

Figure 3. A hypothesized model of the occurrence of pathological events in AD. According to amyloid cascade hypothesis, AD-related pathological cascade is initiated as a result of abnormal increase in Aβ levels or oligomerization. In familial early onset cases (EOAD), the lifelong elevation in Aβ accumulation is caused by mutations in *APP* or *PSEN1* or *-2* genes or *APP* gene duplications. In contrast, a novel protective A673T variant in *APP*, is associated with lifelong decrease in APP processing and Aβ production, and thus reduced risk of late onset AD (LOAD). Down syndrome, which is caused by trisomy of the chromosome 21, on which the *APP* gene resides, is often associated with early onset familial AD (FAD). In LOAD, deficiencies in Aβ clearance, degradation or folding systems are initiated by a complex interplay between genetic and environmental risk factors together with age-related changes. It is also plausible that abnormal tau splicing and/or phosphorylation occur in early stages of LOAD pathology. Increased Aβ levels or misfolding lead to Aβ oligomerization, aggregation and plaque formation. Later hypephosphorylation and aggregation of tau occurs, which leads to NFT formation, synaptic dysfunction, neuronal damage and death and eventually cognitive deterioration and dementia. Neuroinflammation and oxidative stress are also central features of AD pathology.

sufficient to cause FAD (see **Chapter 2.3.1**)(www.AlzGene.org). Furthermore, longitudinal progression studies and biomarker findings in FAD patients imply that Aβ-related changes in the brain and CSF are the first to appear and probably precede the clinical symptoms by decades (**Figure 1**)(Bateman et al., 2012, Jack et al., 2011). Although the vast majority of patients develop AD without these mutations and there is no clear proof of increased Aβ production during LOAD, these disease forms show highly similar neuropathological and clinical manifestations to EOAD. Moreover, data from several studies suggest that malfunction of Aβ clearance and degradation mechanisms could be a common feature of AD and may explain increased Aβ accumulation during LOAD (Baranello et al., 2015). Additionally supporting the amyloid cascade theory, findings from AD animal models indicate that Aβ accumulation precedes and accelerates tau pathology (Eckert et al., 2008, Ferrari et al., 2003, Gotz et al., 2001, Jin et al., 2011, Lewis et al., 2001), while tau is shown to modulate Aβ toxicity (Ittner et al., 2010, Jin et al., 2011, Rapoport et al., 2002, Roberson et al., 2007, Vossel et al., 2010). Based on the strong evidence from epidemiological, genetic and mechanistic studies, Aβ is a key pathological substance in AD, and therefore drugs targeted to halt Aβ accumulation in early stages of the disease have been a logical target for therapeutic intervention for long (Moulder et al., 2013). However, as all therapies targeted towards Aβ have thus far failed to convincingly reduce CSF Aβ levels and have had at best very limited clinical benefits, it seems to be increasingly apparent that reducing Aβ is not necessarily enough to prevent the progression of AD (Coric et al., 2012, Doody et al., 2013, Haapasalo and Kovacs, 2010, Salloway et al., 2009). This has raised a question whether targeting other pathologies centrally involved in AD, such as tau aggregation and hyperphosphorylation, neuroinflammation, or mitochondrial dysfunction might aquire more beneficial effects.

2.2 MOLECULAR MECHANISMS OF ALZHEIMER'S DISEASE

2.2.1 APP and Aβ production

2.2.1.1 APP biochemistry and trafficking

Amyloidogenic Aβ peptides are produced as part of the normal physiological metabolism of APP (Haass et al., 1992b, Haass et al., 1993a). APP is a ubiquitously expressed protein whose physiological function is only partially understood (Findeis, 2007, Zheng and Koo, 2011). The half-life of APP is short. Large quantities of APP are synthesized in the endoplasmic reticulum (ER) and metabolized in other subcellular compartments shortly after (Weidemann et al., 1989, Zheng and Koo, 2011). Newly translated APP is transported from the ER via Golgi apparatus/trans-Golgi network (TGN) to the plasma membrane (PM) through the secretory pathway (Haass et al., 2012, Lichtenthaler, 2012). During its transit, APP undergoes several post-translational modifications to form the mature protein (Weidemann et al., 1989, Zheng and Koo, 2011). From the PM, APP is internalized by a dynamin-dependent endocytosis in clathrin-coated vesicles with the assistance of a 'YENPTY' internalization motif in its C-terminus (Lai et al., 1995). Afterwards, a significant amount of APP is degraded in lysosomes or metabolized in endosomes, while a fraction is targeted to a recycling pathway (Haass et al., 1992a). APP is positioned on a membrane with

its large amino (N) terminal ectodomain at the lumen or extracellular space, the C-terminal tail in the cytosol, and Aβ sequence within the transmembrane domain (Dyrks et al., 1988, Kang et al., 1987). Since APP and all the proteases participating in its processing are membrane-bound proteins, they need to be in close vicinity to form a complex in order for the proteolysis to take place. Thus, subcellular trafficking of these proteins largely defines the path by which APP will be metabolized.

2.2.1.2 APP proteolytic processing

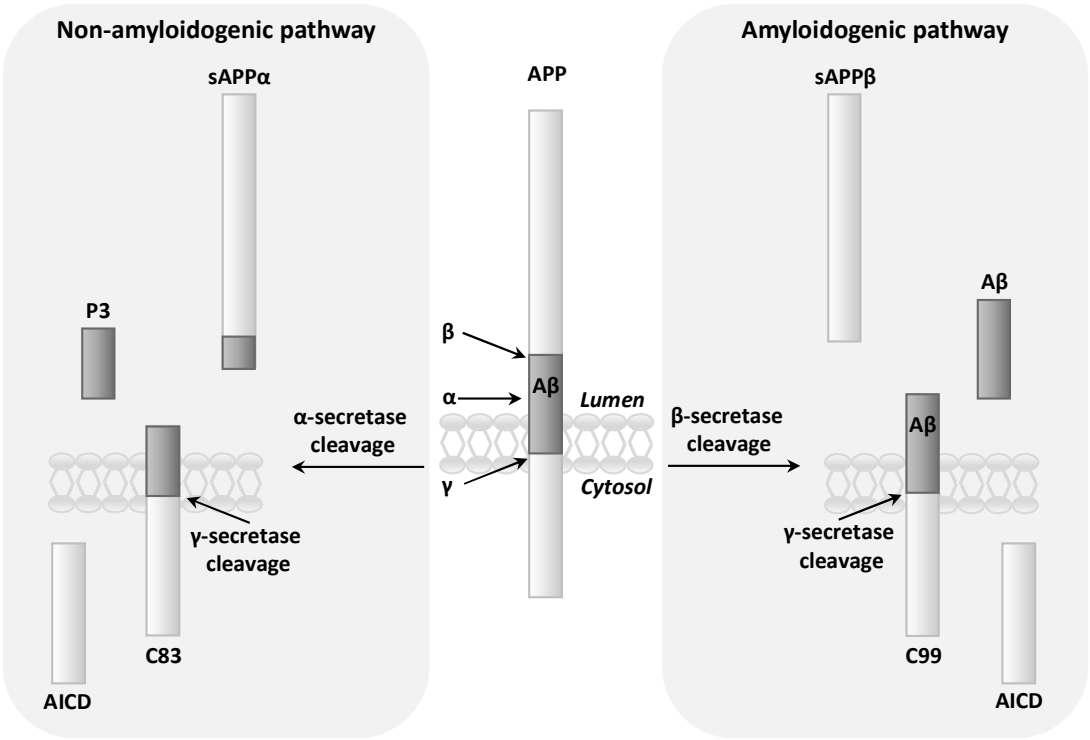

Figure 4. APP proteolytic processing. The non-amyloidogenic processing starts with the cleavage by α-secretase, which leads to generation of soluble APPα (sAPPα) and the C-terminal fragment (CTF), C83. Soluble APPα has been suggested to display neurotrophic and neuroprotective properties as well as reduce BACE1 levels and tau phosphorylation. In the next step, γ-secretase cleaves C83, releasing P3-fragment and the APP intracellular domain (AICD). The function of P3 is not well known, but it has not been found to have neurotoxic effects. AICD is suggested to function in transcriptional regulation of several genes. The amyloidogenic processing is initiated by β-secretase cleavage and leads to the release of sAPPβ and formation of membrane-bound APP CTF, C99. In contrast to sAPPα, sAPPβ may have pro-apoptotic and neurodegenerative effects. Later, C99 is further processed by γ-secretase, which leads to production of AICD and neurotoxic β-amyloid peptide (Aβ), which aggregates into oligomers, fibrils and β-amyloid plaques in the extracellular space.

The proteolytic processing of APP can take two alternative pathways (**Figure 4**). The amyloidogenic route is predominant in neurons and ends up with the release of Aβ. The alternative pathway prevents the formation of Aβ and is prevailent in non-neuronal cells

(Haass et al., 2012). The latter starts with the α-secretase-mediated cleavage on the PM (Haass et al., 2012, Sisodia, 1992). This cleavage is mediated by a group of relatively non-sequence-specific 'A disintegrin and metalloprotease' (ADAM) -family proteases, namely ADAM9, ADAM10, and ADAM17 (Asai et al., 2003, Lichtenthaler, 2012). The α-cleavage blocks Aβ formation by cutting APP in the middle of the Aβ peptide sequence (Sisodia et al., 1990, Sisodia, 1992). Instead, it generates a soluble APPα-fragment (sAPPα) and a membrane bound α-C-terminal fragment (CTFα, C83). In the next phase, γ-secretase cleaves the remaining CTFα at the transmembrane domain, leading to a release of APP intracellular domain (AICD) (Gu et al., 2001) (Sastre et al., 2001) and a truncated Aβ-fragment, called p3 (Haass et al., 1993b). The function of the α-fragments is not fully known, although sAPPα has been suggested to display neurotrophic and neuroprotective properties and lead to reduction of BACE1 levels, subsequent GSK3β inhibition and decreased tau phosphorylation (Deng et al., 2015, Furukawa et al., 1996, Mattson et al., 1993, Meziane et al., 1998, Ring et al., 2007), AICD has been implicated to function in transcriptional regulation (von Rotz et al., 2004), and p3 is assumed to be non-pathogenic (Dulin et al., 2008). Inactivation of the α-secretase has been shown to increase Aβ production, while its overexpression has the opposite effect (Postina et al., 2004). This finding suggests that α-secretase-mediated cleavage may compete with the β-secretase-mediated one, and thus, stimulation of α-secretase could be a potential therapeutic target in AD (Lichtenthaler, 2012, Postina et al., 2004). Interestingly, two rare mutations in *ADAM10*, the principal α-secretase in neurons (Jorissen et al., 2010, Kuhn et al., 2010), were recently identified to co-segregate with LOAD (Kim et al., 2009b). Both mutations were found to disturb the normal folding and function of α-secretase and shift APP processing towards the amyloidogenic pathway. Subsequently, these mutations had the effect of increasing Aβ peptide production, neuroinflammation and plaque burden *in vivo* (Suh et al., 2013). Although this association is yet to be confirmed by others, this information underlines the potential importance of anti-amyloidogenic α-cleavage in AD pathogenesis.

In the β-amyloidogenic processing of APP, it is first cleaved at the N-terminal site of the Aβ sequence by the β-secretase activity (**Figure 4)**. Similar to α-cleavage, β-cleavage releases a soluble APPβ-fragment (sAPPβ) into the lumen or extracellular space and generates a membrane bound CTFβ (C99). In contrast to the sAPPα, the sAPPβ has been shown to have pro-apoptotic effects, cause neurodegeneration and possibly hold other poorly understood toxic features (Nikolaev et al., 2009). In the following step, the CTFβ is further processed by the γ-secretase to generate different Aβ species and the AICD (Dries and Yu, 2008). The γ-secretase is a complex heterotetrameric aspartyl protease, which requires four necessary subunits to form a fully functional enzymatic activity. Presenilin-1 and -2 (PS1 and -2) are the active components of the enzyme containing the catalytic core of the protease (Li et al., 2000, Rogaev et al., 1995, Sherrington et al., 1995, Wolfe et al., 1999), PS enhancer-2 (PEN2) activates and stabilizes the Presenilins, and nicastrin (Nct) and anterior pharynx defective (Aph) -1a or -1b together form an initial scaffold during the assembly of the γ-secretase complex (Francis et al., 2002, Goutte et al., 2002, Yu et al., 2000). Instead of a single site cleavage, CTFβ is processed in a series of stepwise cleavages by the γ-secretase (**Figure 5**) (Haass et al., 2012). In the two alternative paths, the CTFβ is first cleaved after amino acid (aa) 49 or 48 (ε-site cleavage), next at positions aa 46 or 45 (ζ-site cleavage), and finally after any residue located between aa 37 and 43 (γ-site cleavage) (Qi-Takahara et al., 2005, Sastre et al., 2001, Takami et

al., 2009, Weidemann et al., 2002). This cleavage cascade explains the existence of Aβ peptides with various lengths ($A\beta_{37-43}$). While $A\beta_{40}$ is the predominant form of Aβ, $A\beta_{42}$ is more prone to aggregate (Findeis, 2007, Jarrett et al., 1993). Indeed, it has been suggested that an increased ratio of $A\beta_{42}$ to $A\beta_{40}$, rather than the overall Aβ amount is pathologically significant in AD (Findeis, 2007). The generated Aβ peptides are subsequently secreted into the extracellular space between the synaptic terminals. Because of their sticky nature, different extracellular Aβ species form oligomers and fibrils, which are neurotoxic *in vitro* and *in vivo* (Findeis, 2007, Selkoe, 2008).

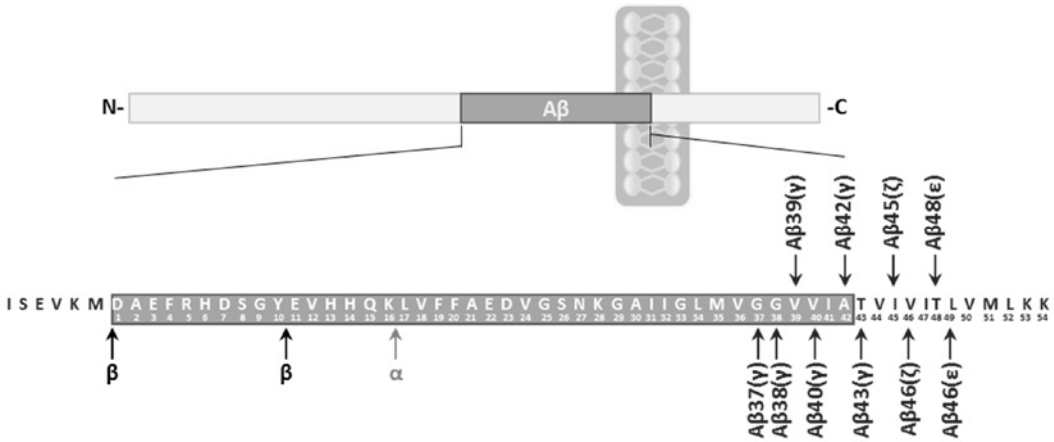

Figure 5. Sequential processing of APP by γ-secretase. The γ-secretase cleaves APP at γ, ζ and ε-sites. Aβ peptides with varying lengths are produced via two main production lines. The predominant production line is initiated with the ε-site cleavage after amino acid 49, followed by ζ-site cleavage after amino acid 46 and finalized by γ-site cleavage after amino acid 43, 40, 38 or 37. $A\beta_{42}$ generation is initiated by ε-site cleavage after amino acid 48, followed by ζ-site cleavage after amino acid 45 and finalized by γ-site cleavage after amino acid 42 or 39. The γ-secretase cleavage sites and the resulting Aβ-peptides are indicated in the APP sequence. Sites for the initiatial α- and β-cleavages are also indicated.

2.2.2 BACE1

2.2.2.1 BACE1, the β-secretase enzyme

The β-secretase-mediated cleavage of APP is the initial and the rate-limiting step in Aβ production. In the late 1990's a transmembrane aspartyl protease called β-site APP cleaving enzyme 1 (BACE1, also known as memapsin or aspartyl protease 2) was identified as being responsible for the β-secretase activity (Hussain et al., 1999, Lin et al., 2000, Sinha et al., 1999, Vassar et al., 1999, Yan et al., 1999). This protein was shown to exhibit all the molecular and functional properties of this secretase and modulation of its levels was shown to have direct consequences on APP processing in cells (Vassar et al., 1999). Given that Aβ production and plaque formation have later been shown to be abolished by *Bace1* knockdown in animal models, it is plausible that BACE1 is also the sole β-secretase enzyme (Cai et al., 2001, Luo et al., 2003, Roberds et al., 2001). Importantly, knockdown of *Bace1* seems to be relatively well tolerated in animals (Luo et al., 2003, Roberds et al., 2001). Because of these promising

findings, BACE1 is currently one of the leading targets in the development of therapies against AD. However, identification and thorough characterization of BACE1 subtrates is pivotal in order to find a safe way to modulate BACE1 in clinical trials. Indeed, recent investigations have revealed several physiological substrates for BACE1, which play key roles central biochemical processes in the nervous system, such as myelination, neurogenesis, neuronal network function, cell adhesion, and axon guidance (Cai et al., 2012, Cole and Vassar, 2007, Hemming et al., 2009, Kuhn et al., 2012, Vassar et al., 2014). Also the ubiquitous nature of BACE1 implies that it may have other yet unidentified physiological substrates (Cheret et al., 2013, Harrison et al., 2003, Hitt et al., 2012, Hu et al., 2015, Laird et al., 2005, Petrus and Lee, 2014, Rajapaksha et al., 2011, Savonenko et al., 2008, Wang et al., 2014).

BACE1 is substantially expressed in the brain and at significantly lower levels in most other tissues. Normally BACE1 protein is highly produced in neurons and to a lesser extent in astrocytes and microglia (Vassar et al., 1999). Several studies have shown that BACE1 protein levels and activity are increased in AD brain as compared to age-matched healthy controls (Fukumoto et al., 2002, Holsinger et al., 2002, Li et al., 2004, Tyler et al., 2002, Yang et al., 2003). In addition, BACE1 levels are upregulated in neurons and astrocytes under different AD-associated stress conditions, such as oxygen and energy deprivation and inflammation (Blasko et al., 2004, Cho et al., 2007, Deng et al., 2014, O'Connor et al., 2008, Tamagno et al., 2002, Velliquette et al., 2005, Wang et al., 2015, Zhang et al., 2007). Mechanistically, stress-induced induction in BACE1 levels may be triggered via transcriptional activation or by post-translational mechanisms (Cole and Vassar, 2007). For instance, HFD-associated inflammation and inflammation induced by the treatment with either lipopolysaccharide (LPS) or proinflammatory cytokine interferon-γ (IFN-γ), has been shown to promote *Bace1* mRNA expression through activation of JAK2 – ERK1/2 – STAT-1 signaling pathway and by binding of NFκB to the *Bace1* gene promoter region in *in vitro* and *in vivo* models (Cho et al., 2007, Wang et al., 2015). On the other hand, elevated BACE1 levels in human AD brain have been proposed to arise from post-transcriptional events, since no alterations in BACE1 mRNA levels have been observed in most studies (Holsinger et al., 2002, Johnston et al., 2005). It has been suggested that increased translation of BACE1 might be mediated by the eukaryotic translation initiation factor 2α (eIF2α), whose activity, under stress, is stimulated by phosphorylation of the amino acid serine at position 51 (O'Connor et al., 2008). Recently, partial silencing of the PERK-eIF2α pathway was shown to reduce BACE1 protein levels, Aβ production, amyloid burden, neurodegeneration, and memory deficits in an AD mouse model (Devi and Ohno, 2014). Another study recently reported that inhibition of monoacylglycerol lipase (MAGL), the primary enzyme that metabolizes the endocannabinoid 2-arachidonoylglycerol (2-AG) in the brain, robustly suppresses BACE1 translation, Aβ formation, neuroinflammation as well as improves both hippocampal synaptic function and spatial learning in an AD mouse model. This is mediated by the activation of PPARγ and NFκB transcription factor signaling pathways and upregulation of the small non-coding RNA, miR-188-3p (Chen et al., 2012, Zhang et al., 2014). Importantly, the expression of miR-188-3p and its murine ortholog has been shown to be decreased in the AD brain and in the brain of AD mice (Zhang et al., 2014). Together, these data point to the idea that different cellular stress conditions may result in increased BACE1 levels which, in

turn, may eventually lead to increased Aβ production and accumulation, further potentiating neuroinflammation as well as neuronal dysfunction and loss during AD developement.

2.2.2.2 BACE1 trafficking and processing

BACE1 is a 501 aa-long type-I transmembrane protein (**Figure 6**)(Vassar et a., 1999). It is initially synthesized in the ER as a zymogen, and, like APP, it traffics from the ER through TNG to the PM via the secretory vesicle pathway (**Figure 7**) (Haniu et al., 2000). In the TGN, BACE1 undergoes N-glycosylation, the catalytic protease domain of BACE1 is folded, and its N-terminal signal sequence and pro-peptide domains are removed to yield the mature enzyme (Benjannet et al., 2001, Capell et al., 2000, Haniu et al., 2000). From the PM, BACE1 is re-internalized by endocytosis, after which it cycles between the Rab5-positive early endosomes, Rab11-positive recycling endosomes, the TGN, and the cell surface, until it is targeted to Rab7-positive late endosomes and lysosomes (LELs) for disposal (**Figure 7**) (Huse et al., 2000, Koh et al., 2005, Pastorino et al., 2002). Besides lysosomes, BACE1 can be degraded via the ubiquitin-proteasome system (UPS), autophagy and by endoproteolysis (Huse et al., 2000, Qing et al., 2004, Wu et al., 2015).

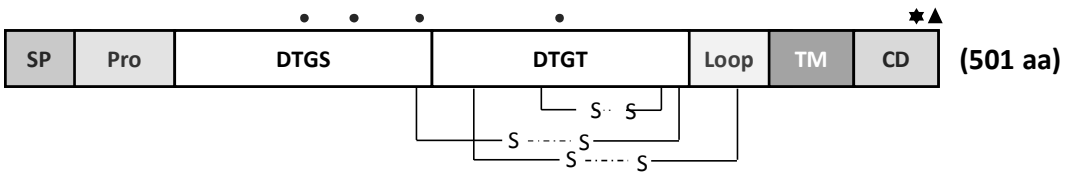

Figure 6. Schematic presentation of BACE1 protein structure. BACE1 is a 501 amino acids long protein, which contains a signal peptide (SP) and a pro-domain (Pro) in the N-terminus, two consensus motifs, DTGS and DTGT, in the protease domain and loop-domain, transmembrane domain (TM) and cytosolic domain (CD) at the C-terminus. Glycosylation sites are indicated with black circles, phosphorylation site with black asterisk and ubiquitination site with black triangle above the protein structure. Three disulfide bonds (S–S) are present in the folded protein. aa, amino acid.

Endocytosis of BACE1 occurs through a mechanism that is distinct from that undergone by APP, and these two proteins are only united in clathrin-coated vesicles or at early endosomes (Vassar et al., 2014). In particular, early endosomes, have the optimal pH of ~4.5 for β-secretase activity, making them a major site for Aβ production (Ehehalt et al., 2003, Kalvodova et al., 2005, Rajendran et al., 2006, Sannerud et al., 2011). However, some of BACE1 activity has also been reported in other acidic compartments, including the TGN. It is noteworthy that a causative 'Swedish' mutation in *APP* (APPswe) leads to a more efficient cleavage of APP by BACE1, particularly in the TGN (Haass et al., 2012). This implies that increased BACE1-mediated cleavage of APP via an altered subcellular localization of APP and BACE1 interactions may be sufficient to cause AD.

In membraneous compartments, BACE1 localizes to lipid rafts and this is at least partly regulated by the palmitoylation of BACE1 (Ehehalt et al., 2003, Kalvodova et al., 2005, Riddell et al., 2001, Vetrivel et al., 2009). Lipid rafts are cholesterol and sphingolipid-enriched membrane microdomains, which are thought to be the focal points for the amyloidogenic

APP processing at the PM (Cordy et al., 2003, Ehehalt et al., 2003). Conversely, non-amyloidogenic APP processing likely occurs outside the lipid rafts as the α-secretase is localized in the non-raft regions of the memrane (Kojro et al., 2001). Several membrane lipids have been shown to regulate raft localization of BACE1 and stimulate its activity *in vitro* (Kalvodova et al., 2005). Interestingly, lipid rafts derived from AD brains were recently found to exhibit altered lipid profiles and associate with a facilitated interaction between APP and BACE1 as compared to control subjects (Diaz et al., 2015, Fabelo et al., 2014). Together these data point to a critical role of certain lipids in amyloidogenic processing of APP.

Subcellular sorting of BACE1 is regulated by several factors. Endocytosis and sorting into early endosomes is regulated by ADP ribosylation factor 6 (ARF6) (Sannerud et al., 2011).

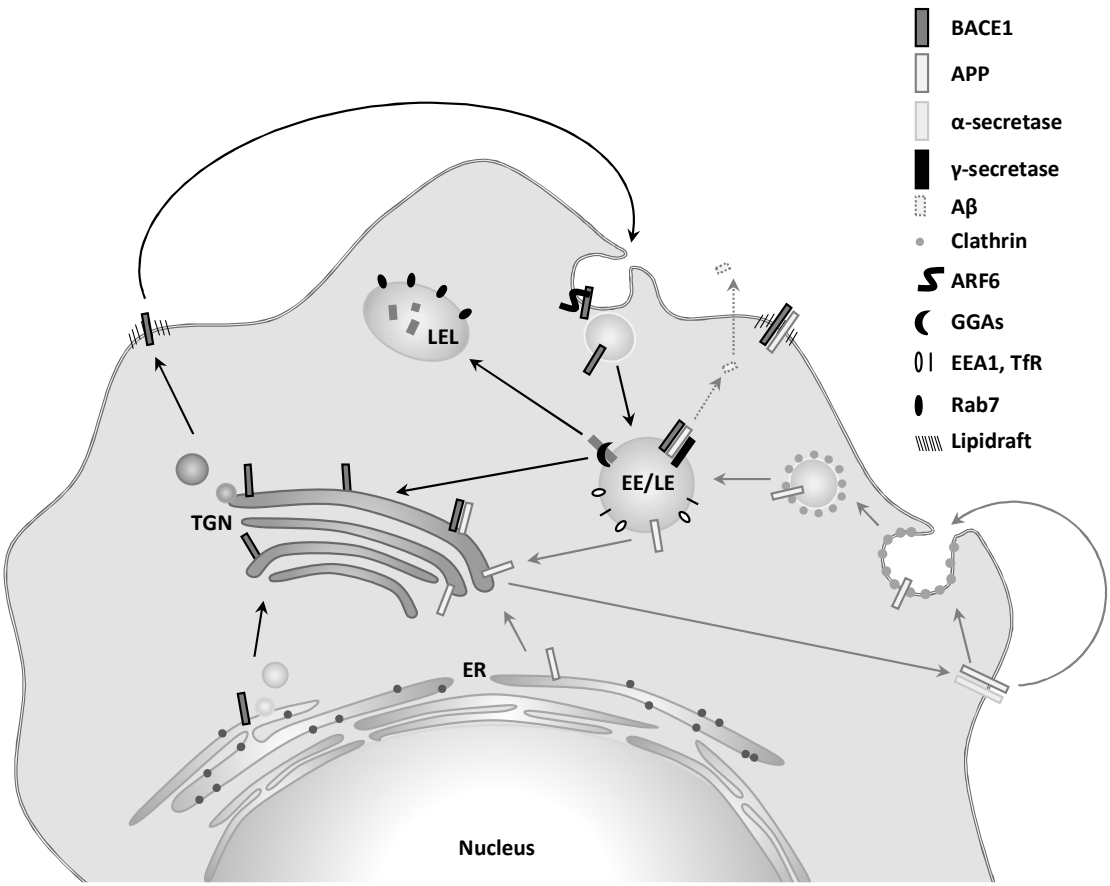

Figure 7. Schematic presentation of BACE1 and APP subcellular trafficking. BACE1 traffics from endoplasmic reticulum (ER) via trans-Golgi network (TGN) to plasma membrane (PM). BACE1 is re-internalized via ARF6-mediated endocytosis and directed to recycling pathway or late endosomes/lysosomes (LELs) for degradation. APP traffics from ER to TGN and PM and is re-internalized in clathrin-coated vesicles. Potential sites for APP and BACE1 interaction are lipid rafts, early and late endosomes (EE/LE) and TGN. BACE1 trafficking is indicated with black arrows. APP trafficking is indicated with grey arrows.

BACE1 sorting into LELs is reduced by addition of N-acetyl-D-glucosamine (GlcNAc) sugar moieties, which recently was shown to be increased in the AD brain (Kizuka et al., 2015). In animals, inhibition of GlcNAcylation was found to direct BACE1 into LELs, which reduced its co-localization with APP, subsequently decreasing Aβ production, plaque burden and cognitive deficits (Kizuka et al., 2015). Sorting of BACE1 in endosomes and lysosomes is also controlled by the carboxyl-terminal sorting signals, such as acidic di-leucine motifs and addition of ubiquitin at Lys501 (Huse et al., 2000, Kang et al., 2012, Koh et al., 2005, Pastorino et al., 2002). These signal motifs are recognized by adaptor proteins, called Golgi-localized γ-ear containing ADP ribosylation-binding factor (GGA) -1, -2, and -3 (Kang et al., 2010, Tesco et al., 2007), Phosphorylation of BACE1 at Ser498 seems to regulate this interaction (Pastorino et al., 2002, Walter et al., 2001). Several studies suggest that GGAs retarget BACE1 from endosomes towards the TGN (GGA1) or lysosomes for degradation (GGA3) (Cole and Vassar, 2007, Kang et al., 2010, Tesco et al., 2007). As a consequence, GGAs shorten the time of endosomal co-localization of APP and BACE1, which subsequently may reduce Aβ production. In line with this, depletion of GGA3 has been shown to increase BACE1 levels and activity by reducing its lysosomal targeting and degradation (Kang et al., 2010, Sarajarvi et al., 2009, Tesco et al., 2007). Interestingly the levels of GGA1, GGA3 and DHCR24/Seladin-1, a protein regulating GGA3 levels during stress, have been shown to be decreased in AD brain (Greeve et al., 2000, Kang et al., 2010, Sarajarvi et al., 2009, Tesco et al., 2007). Altogether, these data imply that subcellular sorting of BACE1 may have a pivotal role in Aβ generation. Therefore, a comprehensive understanding of BACE1 intracellular trafficking mechanisms may provide new insights into AD pathogenesis and therapeutic strategies.

2.2.3 Tau

2.2.3.1 Tau structure and expression

The human tau encoding gene (*MAPT*) is located on the chromosome 17q21 and the full-length protein is encoded by 13 exons. Six major tau isoforms are produced by the alternative splicing of three exons (**Figure 8**) (Goedert et al., 1989). The C-terminal part of the protein has a microtubule-binding domain, which includes four repeat-sequences encoded by exons 9, 10, 11 and 12. The alternative inclusion or exclusion of exon 10 (2nd repeat) leads to the expression of three tau isoforms with four repeat-sequences (4R-tau) and three isoforms containing only three repeat-sequences (3R-tau). These 4R-tau and 3R-tau isoforms differ with respect of the presence of N-terminal exons 2 and 3. As a consequence, each 4R-tau and 3R-tau isoform may either include both exons 2 and 3 (2N4R and 2N3R), only exon 2 (1N4R and 1N3R) or neither of them (0N4R and 0N3R). Besides the microtubule binding motif on its C-terminus and the "projection domain" on its N-terminus, tau also contains a proline-rich domain in the middle region. The microtubule-binding domain interacts with microtubules, whereas the projection domain projects away from them. The proline-rich domain functions as a binding site for proline-directed kinases and other proteins with SRC-homology 3 (SH3) –domain (Mandelkow and Mandelkow, 2012).

The expression of tau isoforms in the central nervous system (CNS) is strictly regulated. Whereas only the shortest 0N3R-tau isoform is expressed during embryogenesis, the ratio of 4R-tau and 3R-tau isoforms is maintained at equality in adult humans (Goedert et al., 1989, McMillan et al., 2008) (Kosik et al., 1989). The one-to-one ratio of 3R-tau to 4R-tau seems to be essential for maintaining normal brain function, while disregulation of exon 10 splicing is linked to neurodegenerative disorders. Several familial *MAPT* genetic mutations are associated with neurodegenerative tauopathies (diseases that display abnormal levels or forms of the protein) lead to abnormal exon 10 splicing and thus an imbalance between 4R-tau and 3R-tau isoforms (Lee et al., 2001). Although the role of aberrant tau splicing is a matter of debate in AD, it has been demonstrated that increased levels of 3R tau may play a role in the progression of tau pathology during AD (Connell et al., 2005, Espinoza et al., 2008, Glatz et al., 2006, Ingelsson et al., 2006). This idea is supported by the finding that the early onset tau pathology in individuals with Down syndrome (trisomy 21) may be associated with a relative increase in 3R-tau expression (Shi et al., 2008). Several serine/arginine-rich splicing factors (Srsfs), including Tra2β, ASF/SF2, SC35, Srp30c and Srp55, act on alternative splicing of tau exon 10 and their function is regulated via phosphorylation by a number of kinases, such as GKS3β, PKA and Dyrk1A (Liu and Gong, 2008, Qian and Liu, 2014). Interestingly, Aβ has been shown to regulate GSK3β-mediated activation of SC35, whereas Dyrk1A is

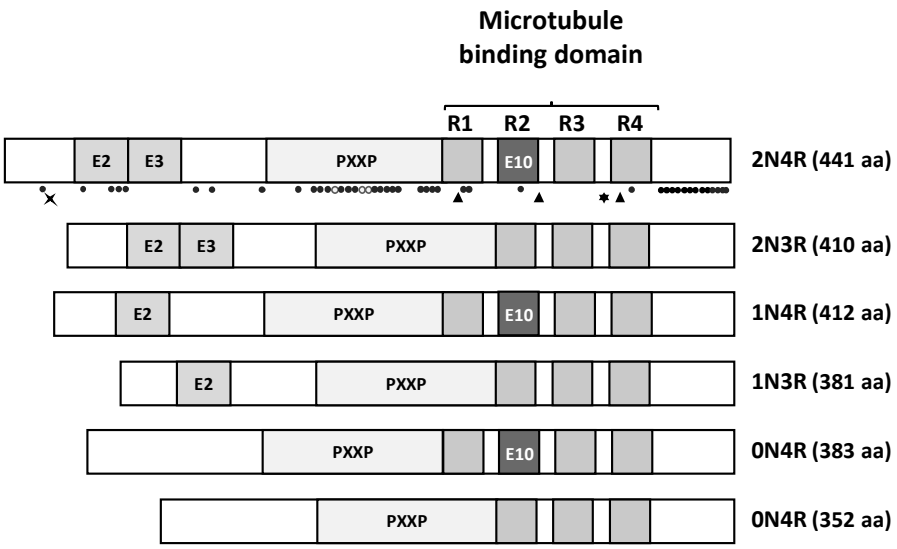

Figure 8. Illustration of tau protein structure, modifications and isoforms. Six major isoforms are created by alternative splicing of N-terminal exons 2 (E2) and 3 (E3) and C-terminal exon 10 (E10). C-terminal microtubule-binding domain contains either four (4R) or three (3R) repeat-sequences, depending on the presence of exon 10, encoding the 2nd repeat. Two (2N), one (1N) or none (0N) of the N-terminal inserts can be included in the protein. The proline-rich (PXXP) domain is located in the middle of the protein and mediates the interaction with specific protein domains. Phosphorylation sites are indicated with black circles. The sites (S191, S202/T205) specific for AT8 antibody, often used to assess AD-related tau phosporylation, are indicated with grey circles. Sumoylation site (L340) is indicated with an asterisk, nitration site (T29) is indicated with a star. OGlcNAc occurs on several S and T residues.

upregulated in Down syndrome (Chen et al., 2010, Shi et al., 2008). Furthermore, altered expression of Tra2β and Srp55 has been observed in the tissues of obese humans and rodents and a recent study reported that chronic T2DM in obese rats alter tau exon 10 splicing, so that 3R-tau isoforms become overrepresented (Jiang et al., 2003)(Jung et al., 2011, Kondo et al., 2004, Liu and Gong, 2008, Pihlajamaki et al., 2011). Since obesity and TD2M are risk factors for AD, these data imply that these metabolic factors may predispose to AD by shifting the balance between the different tau isoforms. In the functional point of view, 4R-tau is known to interact with microtubules more strongly than 3R-tau and thus diverse tau isoforms may differentially affect microtubule dynamics and function (Trinczek et al., 1995). Another recent paper suggested that a plausible mechanism, for the underlying neurodegeneration during abnormal tau splicing, is related to impaired endosomal vesicle trafficking and increased cellular stress (Wren et al., 2015). In summary, the imbalance between tau isoforms seems to be sufficient to cause neurodegeneration.

2.2.3.2 Tau function and post-translational regulation

Tau is expressed in several tissues, but it is most abundant in the axons of CNS neurons where the distances are long and the significance of microtubular integrity cannot be understated (Goedert et al., 1989, Gu et al., 1996). Indeed, the primary function of tau is believed to stand on the promotion and stabilization of microtubule assembly as well as on microtubule-associated axonal transport of different molecular cargo (Lee and Leugers, 2012). Tau shares a significant structural homology with two other family members of microtubule-associated proteins (MAPs), MAP2 and MAP4 (Chapin and Bulinski, 1991). The evidence from tau knockout animal models lend support to the fact that the some of the functions of tau may be compensated by the other MAPs. Despite this functional redundancy, however, only tau has been associated with neurodegenerative diseases, which clearly illustrates that the different MAPs are likely to have very distinct roles (Grundke-Iqbal et al., 1986, Kosik et al., 1986).

Tau is post-translationally modified by a number of different moeities, including phosphorylation, O-linked GlcNAcylation, nitration, ubiquitination, and sumoylation (**Figure 8**). Phosphorylation of tau at dozens of individual residues is a strictly regulated dynamic process, which has an important physiological role. Fetal tau is significantly more phosphorylated than the adult tau, suggesting that phosphorylation has a role in neuronal development (Lee and Leugers, 2012). Phosphorylation of adult tau defines how tightly tau can be bound to microtubules and leads to conformational changes in tau, which may alter its ability to interact with other proteins. However, the most important evidence regarding the critical role of tau phosphorylation arises from the fact that abnormal hyperphosphorylation of tau precedes its aggregation of into NFTs and similar deposits in AD, FTD, and other neurodegenerative diseases. In AD, tau is phosphorylated at sites that are normally unphosphorylated in adult tau or exceedingly phosphorylated at the sites that are normally phosphorylated (Grundke-Iqbal et al., 1986). For instance, strong phosphorylation of tau at epitopes recognized by the AT8 antibody is detected during neuronal development, but also prior to tau aggregation in AD pathogenesis (Lee ang Leugers, 2012). Abnormal tau phosphorylation is also commonly observed in animal models with experimentally induced diabetes and this has been found to associate with decline in

behavioral tasks (Kim et al., 2009, Li et al., 2007, Planel et al., 2007, Shcubert et al., 2003). Furthermore, it has been shown that tau is transiently hyperphosphorylated during hibernation and anesthesia-associated hypothermia, a condition which is often followed by temporal deterioration and cognitive decline. These studies together suggest that tau phosphorylation level is altered during several different physiological and pathological conditions (Avila et al., 2012, Planel et al., 2007). It has been shown that many kinases, such as GSK3β, cdk5, MAPK (ERK), JNK, MARK, PKA, CK1, CaMKII, PKC, Fyn, and Src, and phosphatases, including protein phosphatase 1 and 2A (PP1 and PP2A, respectively), act on tau phosphorylation sites. Of these, at least GSK3β and MAPK are involved in aberrant tau phosphorylation during AD, whereas PP2A has been shown to associate with abnormal tau phosphorylation in a subgroup of FTD (Goedert et al., 2000). Because O-GlcNAc competes with phosphorylation for the same sites on the same residues, it has been suggested to indirectly regulate tau phosphorylation (Liu et al., 2002). Furthermore, tau ubiquitination as well as nitration (which is a reaction catalyzed by reactive nitrogen species during oxidative stress) have been found in AD and other neurodegenerative tauopathies (Cripps et al., 2006, Reynolds et al., 2006). Altogether, these studies imply that post-translational modifications are important regulators of normal tau function and pathology. Besides post-translational covalent modifications, accumulating evidence from recent studies suggest that pathological tau is abnormally truncated and possesses an aberrant conformation. These truncated and misfolded tau species can be internalized by nearby neurons, probably via synaptic contacts. This provides an explanatory mechanism by which tau pathology propagates through the brain areas during disease progression, spreading dysfunction (Calafate et al., 2015, Matsumoto et al., 2015, Medina and Avila, 2014, Sokolow et al., 2015).

2.2.4 Ubiquilin-1

2.2.4.1 Ubiquilin-1 structure, function and interactome

Several proteins that have been identified as regulators of protein folding, processing, subcellular targeting and degradation, have also been linked to the pathogenesis of certain neurodegenerative diseases. One such protein is ubiquilin-1 [also known as PLIC-1 (protein linking integrin-associated protein with cytoskeleton-1 (Haapasalo et al., 2010). Ubiquilin-1 belongs to a highly conserved group of ubiquitin-like proteins, which deliver polyubiquitinated proteins to the proteasome for degradation (Kleijnen et al., 2003, Ko et al., 2002, Mah et al., 2000, Marin, 2014). In humans, Ubiquilin-1 is encoded by the *UBQLN1* gene, which consists of eleven exons. The full-length ubiquilin-1 protein contains two characteristic functional protein domains: the N-terminal ubiquitin-like domain (UBL) mediates the interaction with the 26S proteasome by directly binding to S5a-component of the 19S proteasomal subunit, and the C-terminal ubiquitin-associated domain (UBA) that cooperates with poly-ubiquitin chains attached to the proteins destined for degradation (**Figure 9**). The presence of these highly specified domains indicates that ubiquilin-1 functions as a shuttle between the proteasome and misfolded or accumulated proteins (Kleijnen et al., 2003, Ko et al., 2002, Mah et al., 2000). A central region of ubiquilin-1 consists of conserved asparagine- and proline-rich (Asn-Pro-rich) repeats. These repeats interact with specific domains of other proteins, such as epidermal growth factor substrate 15 homology (EH) –domain that is

present in a number of proteins that regulate endocytosis and vesicle sorting, suggesting that ubiquilin-1 may be involved in intracellular vesicular trafficking (Mah et al., 2000).

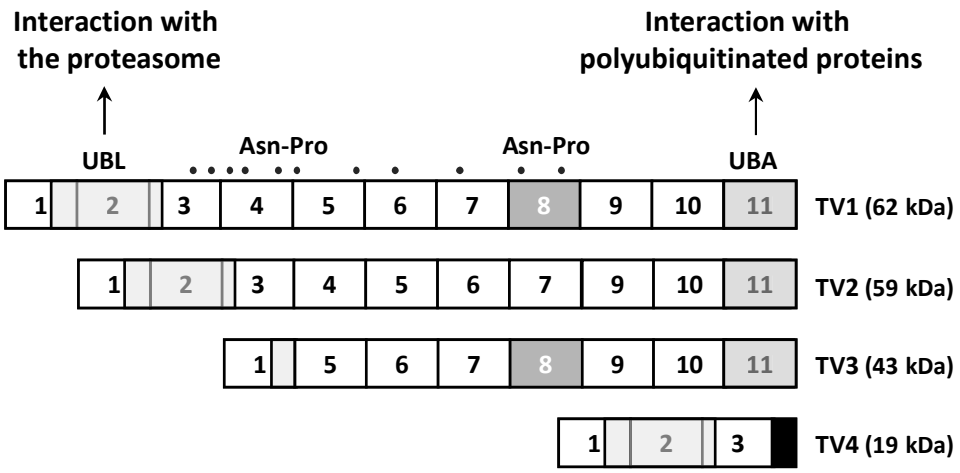

Figure 9. Schematic representation of ubiquilin-1 protein structure and transcript variants (TVs). The full length ubiquilin-1 protein, TV1, is encoded by 11 exons of the *UBQLN1*. Ubiquilin-1 has a ubiquitin-like domain (UBL) in the N-terminus, which interacts with the S5a domain of the 19S proteasomal subunit. The C-terminus contains the ubiquitin-associated domain (UBA), which mediates the interaction with polyubiquitinated proteins. The TV2 lacks the exon 8. Exons 2, 3 and 4 are excluded from the TV3. The TV4 protein is encoded only exons 1, 2 and 3 and has a unique C-terminus. Asparagine and proline (Asn-Pro) -rich repeats are indicated with black circles.

Ubiquilin-1 is ubiquitously expressed in most, if not all, tissues (Marin, 2014). In the human brain, Ubiquilin-1 is present in neurons and inside the cells it localizes to the cytoplasm, ER, and to a lesser extent in the nucleus and peripheral parts of the cell (Mah et al., 2000). Four alternatively spliced*UBQLN1* gene derived transcript variants (TVs) have been identified in human brain (**Figure 9**) (Bertram et al., 2005, Lu et al., 2009). These TVs encode four protein isoforms, which differ in their domain composition, implying that they might have unique interactomes and functions (Haapasalo et al., 2011). Compared to TV1, which is the full-length form of ubiquilin-1, TV2 lacks the exon 8, encoding parts of Asn-Pro-rich repeats. TV2 may therefore have an altered ability to interact with certain proteins. TV3 does not harbor exons 2, 3, and 4, and thus the TV3 protein contains only a fraction of the N-terminal UBL domain. This may prevent the binding to the proteasome and thus this isoform may not be able to efficiently deliver polyubiquitinated proteins for proteasomal degradation. The smallest isoform, TV4, is encoded by only exons 1, 2, and 3, and this protein has a unique 32-aa insertion in its C-terminus. Consequently, TV4 completely lacks the C-terminal UBA domain and a large part of Asn-Pro-rich repeats, which may interfere with its ability to interact with poly-ubiquitinated and other protein targets. However, the physiological functions of individual isoforms are not well known (Haapasalo et al., 2011).

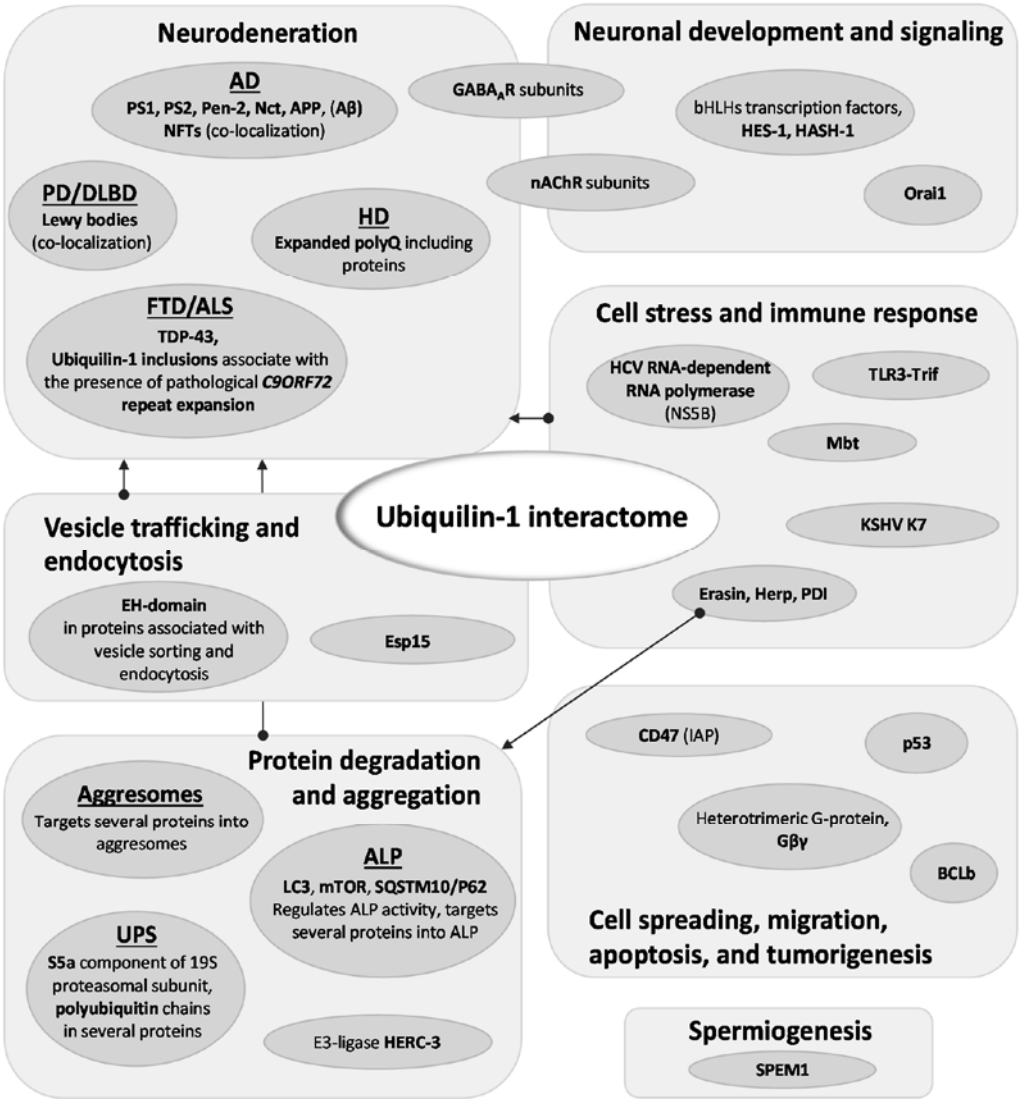

Figure 10. Schematic representation of suggested protein interactions and functions of ubiquilin-1. Ubiquilin-1 interacts with numerous proteins, which have a role in neurodegeneration, neuronal development and signaling, vesicle trafficking and endocytosis, cell stress and immune response, protein aggregation and degradation, cell spreading, migration, apoptosis and tumorigenesis as well as spermiogenesis. AD, Alzheimer's disease; ALP, autophagosome and lysosome pathaway; ALS, amyotrophic lateral sclerosis; APP, amyloid precursor protein; BCLb, B-cell lymphoma; bHLHs, basic helix-loop-helix transcription factors; CD47/IAP, integrin-associated protein; *C9ORF72*, Chromosome 9 open reading frame 72 encoding gene; DLBD, diffuse Lewy body disease; EH, epidermal growth factor substrate 15 homology; Earsin and Herp, ER-resident proteins; Esp15, epidermal growth factor receptor pathway substrate protein 15; FTD, frontotemporal degeneration; GABA$_A$R, γ-aminobutyric acid type A receptor; HERC-3, HECT and RLD domain-containing E3 ubiquitin protein ligase 3; HCV, hepatitis C virus; KSHV, Kaposi's sarcoma-associated herpes virus; LC3, microtubule-associated protein 1A/1B-light chain 3; Mbt, mycobacterium tuberculosis; mTOR, mammalian target of rapamycin; nAChR, nicotinic acetylcholine receptor; Nct, nicastrin; NFT, neurofibrillary tangle; Orai1, calcium release-activated calcium channel protein 1; Pen-2, presenilin enhancer-2; PDI, protein disulfideisomerase; PolyQ, polyglutamine expansion proteins; PS1, PS2, presenilin-1, -2; SPEM1, spermatid maturation; SQSTM1/P62, sequestosome 1; TLR3-Trif, Toll-like receptor-Trif; UPS, ubiquitin-protesome system.

Functional studies in *in vitro* and *in vivo* models have proven that ubiquilin-1 regulates the trafficking, function, levels and degradation of numerous proteins (**Figure 10)**. The diversity of the ubiquilin-1 interactome suggests that it is involved in a variety of physiological and related pathophysiological functions. In addition to proteasomal targeting, several studies have indicated that ubiquilin-1 may also mediate the clearance of cellular waste and pathogens via the autophagosome-lysosome pathway (ALP) (N'Diaye et al., 2009, Rothenberg et al., 2010, Sakowski et al., 2015, Viswanathan et al., 2011, Wu et al., 2002). Ubiquilin-1 by itself is a target for ALP and it has been suggested to interact with autophagosomes through the UBA domain (Rothenberg et al., 2010, Viswanathan et al., 2011). When protein accumulation exceeds the capacity of the UPS or ALP, ubiquilin-1 has been shown to target unwanted proteins into intracellular inclusion bodies, termed aggresomes (Johnston et al., 1998, Kopito, 2000, Massey et al., 2004, Olzmann et al., 2008, Viswanathan et al., 2011). Structurally, these juxtanuclear inclusions closely resemble characteristic intracellular inclusions containing aggregated proteins associated with AD and other neurodegenerative diseases. Accumulating evidence implies that these structures may be cytoprotective, since they sequester potentially harmful proteins into restricted compartments, which may later be safely disposed through the ALP (Haapasalo et al., 2010) (Haapasalo et al., 2011).

2.2.4.2 Ubiquilin-1 and cell stress

Several studies provide evidence that ubiquilin-1 plays a role during different cellular stress conditions (**Figure 10**)(Lim et al., 2009, Liu et al., 2014, Lu et al., 2009, N'Diaye et al., 2009) (Kim et al., 2008). Ubiquilin-1 protects cells from starvation-induced apoptosis in an autophagy-dependent mechanism (N'Diaye et al., 2009). Moreover, the levels of ubiquilin-1 are up-regulated during the unfolded protein response (UPR) and it has been shown to protect cells from ER-stress-associated apoptotic cell death (Ko et al., 2002, Lim et al., 2009, Lu et al., 2009). All ubiquilin-1 TVs, except TV4, have been shown to reduce the induction of UPR-inducible stress genes, subsequently leading to enhanced cell survival during ER-stress and hypoxia (Lu et al., 2009). The beneficial effect of ubiquilin-1 during acute stress has been suggested to occur by enhancing the proteasomal disposal of ER-associated degradation (ERAD) substrates (Kim et al., 2008, Lim et al., 2009). Supporting this idea, ubiquilin-1 down-regulation *in vivo* in *Caenorhabditis elegans* and in mice has been found to result in the accumulation of misfolded and polyubiquitinated proteins during induced ER-stress, oxidative stress and ischemia (Lim et al., 2009, Liu et al., 2014). Accordingly, ubiquilin-1 overexpression was recently reported to protect mice from oxidative stress and ischemic stroke-induced neuronal injury and motor defects (Liu et al., 2014). Together this data indicate that ubiquilin-1 may protect cells from stress by inhibiting the accumulation of damaged proteins.

2.2.4.3 Ubiquilin-1 and protein aggregation in neurodegeneration

During recent years, ubiquilin-1 has been linked to AD pathogenesis in many ways (**Figure 10**). Several studies have demonstrated that certain genetic variants in *UBQLN1* are associated with an increased risk of developing AD(Bertram et al., 2005, Golan et al., 2008, Kamboh et al., 2006, Slifer et al., 2005, Yue et al., 2015), although not all studies have been

able to replicate this association (Arias-Vasquez et al., 2007) (Slifer et al., 2006) (Bensemain et al., 2006, Brouwers et al., 2006, Martin et al., 2012, Smemo et al., 2006). Supporting the genetic data, the risk allele of UBQ-8i single nucleotide polymorphism (SNP) has been reported to alter the mRNA ratio of TV2 to TV1 and cause a pathological phenotype *in vivo* in the fruit fly model organism, *Drosophila melanogaster* (Ganguly et al., 2008, Gross et al., 2008). In a recent study, ubiquilin-1 protein levels were reported to be significantly decreased in the brain of LOAD patients (Stieren et al., 2011). Although this was not associated with any particular *UBQLN1* genotype, this data implies that ubiquilin-1 function may be diminished during AD. On top of that, mutations in *UBQLN2* (ubiquilin-2 gene), which encodes a protein highly homologous to ubiquilin-1, have been recently shown to cause rare familial amyotrophic lateral sclerosis (ALS), a devastating disease affecting upper and lower motoneurons (Deng et al., 2011). Although these mutations are not associated with AD, this finding further underlines the role of ubiquilin family proteins in neurodegenerative disorders.

From the functional point of view, ubiquilin-1 was originally identified as a PS1 and -2 interacting protein, which later has been demonstrated to specifically increase the accumulation and aggresomal targeting of ubiquitinated high-molecular weight (HMW) PS-complexes in several studies (Ganguly et al., 2008, Li et al., 2007, Mah et al., 2000, Massey et al., 2004, Massey et al., 2005, Thomas et al., 2006, Viswanathan et al., 2011, Zhang and Li, 2015). This function of ubiquilin-1 may partially be isoform-dependent, since TV3, which lacks the proteasome-interacting UBL domain, has been found to enhance PS1 accumulation and aggresomal localization (Viswanathan et al., 2011). However, the possible functional consequences of the interrelationship between ubiquilin-1 and the presenilins are yet to be discovered. In addition to PSs, ubiquilin-1 also seems to modulate the levels of other γ-secretase components, Pen-2 and Nct, but the effects of this interaction have been inconclusive (Massey et al., 2005, Viswanathan et al., 2011). Importantly, number of studies have reported that modulation of ubiquilin-1 levels in cultured cells alters APP processing, maturation, trafficking and proteolysis, consequently affecting Aβ production and secretion in a PS/γ-secretase-independent mechanism (El Ayadi et al., 2012b)(Hiltunen et al., 2006, Stieren et al., 2011, Viswanathan et al., 2013, Zhang et al., 2007). Accordingly, the data regarding ubiquilin-1 effects on APP in *Drosophila melanogaster* have led to the proposition that ubiquilin-1 plays a role in APP processing also *in vivo* (Gross et al., 2008, Li et al., 2007). However, as these data have been fairly inconsistent and suggested that both downregulation and overexpression may increase APP maturation, aggregation, and processing, this interrelationship may be cell-type-specific and needs to be confirmed in additional models. Nevertheless, the mechanistic studies suggest that ubiquilin-1 functions as an important regulator of PS1- and 2 levels and as a molecular chaperone for APP. Therefore, it is plausible that dysregulation of ubiquilin-1 levels or function in AD brain may have consequences on Aβ accumulation.

Finally, ubiquilin-1 has been shown to localize in NFTs, dystrophic neurites, and Hirano bodies in the AD brain, and in Lewy bodies in Parkinson's disease (PD) brain, as well as diffuse Lewy body disease (DLBD) (Mah et al., 2000, Mizukami et al., 2014, Satoh et al., 2013). A recent study reported that early NFT changes are associated with upregulation or nuclear translocation of ubiquilin-1 in hippocampal neurons (Mizukami et al., 2014). However,

further studies unraveling the possible relationship between tau and ubiquilin-1 have not been published so far. Besides AD and PD, ubiquilin-1 has been found to regulate the accumulation and toxicity of expanded polyQ repeat containing proteins, such as HTT in Huntington's disease (HD) and polyA containing proteins associated predominantly with congenital malformation syndromes (Albrecht and Mundlos, 2005)(Doi et al., 2004, Safren et al., 2014, Wang and Monteiro, 2007a, Wang and Monteiro, 2007b). Recently, ubiquilin-1 was also reported to localize in the ubiquitin/sequestosome-1 (SQSTM1/p62) and TAR DNA-binding protein 43 (TDP-43) –immunopositive intracellular inclusions in FTD and ALS brain and mediate the stability and toxicity of these aggregates *in vitr*o and *in vivo* (Brettschneider et al., 2012, Hanson et al., 2010, Kim et al., 2009c). Interestingly, ubiquilin pathology in FTD and ALS brains seems to strongly associate with the presence of a pathological *C9ORF72* (C9orf72 gene)-associated genotype. The hexanucleotide repeat expansion located in this recently identified gene is the most common genetic cause of both FTD and ALS (DeJesus-Hernandez et al., 2011)(Renton et al., 2011). The C9orf72 protein was recently proposed to regulate the endosomal trafficking system, which is necessary for the controlling, sorting and degradation of proteins via the ALP or the UPS, implying a possible mechanistic link between C9orf72 and ubiquilin-1 (Korolchuk et al., 2010, Levine et al., 2013) (Farg et al., 2014). Taken together, ubiquilin-1 regulates several essential cellular functions and participates in aggregation and degradation of numerous proteins abnormally accumulating in neurodegenerative diseases. This suggests that ubiquilin-1 might represent a common mechanistic link between distinct neurodenerative diseases. However, whether ubiquilin-1 augments or alleviates the pathology in these diseases, remains an open question.

2.3 THE EFFECT OF GENES AND ENVIRONMENT ON ALZHEIMER'S DISEASE

2.3.1 Genetics of Alzheimer's disease

2.3.1.1 Causative mutations in APP, PSEN1, and PSEN2

The familial component is the second most important risk factor for AD after age. It is estimated that heritability in AD may be as high as 80% (Gatz et al., 2006). However, only in rare cases (<1%) is AD inherited in a fully penetrant, autosomal dominant, fashion. These early onset familial AD (FAD) cases are caused by mutations in three genes: *APP*, *PSEN1*, and *PSEN2*, which encode for the APP, PS1, and PS2 proteins, respectively (**Table 1**) (www.AlzGene.org, De Strooper et al., 1998, Glenner and Wong, 2012, Kang et al., 1987, Masters et al., 1985, Selkoe, 2001, Wolfe et al., 1999). The vast majority of causative mutations reside in *PSEN1* gene, and a few in the homologous gene *PSEN2*. All *PSEN1* and *PSEN2* mutations increase the ratio of $A\beta_{42}$ to $A\beta_{40}$, by poorly understood mechanisms, thus subsequently promoting Aβ oligomerization and formation of amyloid fibrils (Borchelt et al., 1996, Chavez-Gutierrez et al., 2012, Jarrett et al., 1993, Potter et al., 2013, Weggen and Beher, 2012). In addition, there are more than 30 mutations in *APP*, each located within the Aβ sequence or in the close vicinity of the β- or γ-secretase cleavage sites (Ringman et al., 2014). Depending on the exact location, *APP* mutations either promote Aβ oligomerization (such as the Arctic E693G mutation within Aβ sequence) (Nilsberth et al., 2001), increase the relative amount of $A\beta_{42}$ (such as London mutation located just beyond the C-terminus of Aβ) (Suzuki

et al., 1994), or elevate BACE1 affinity towards APP and result in an overall increased total Aβ production (such as Swedish KM670/671NL mutation near the β-secretase cleavage site) (Mullan et al., 1992, Weggen and Beher, 2012). Besides these gene mutations, copy number variations, such as *APP* gene duplications and chromosome 21 trisomy (Down syndrome) on which *APP* resides, cause autosomal dominant early onset AD (Rovelet-Lecrux et al., 2006, Rumble et al., 1989, St George-Hyslop et al., 1987a, St George-Hyslop et al., 1987b, Tanzi et al., 1987a, Tanzi et al., 1987b). Taken together, all these data imply that lifelong exposure to increased Aβ levels is sufficient to cause FAD and therefore the imbalance in Aβ levels is likely to be a defining molecular event in AD. This concept recently gained further support as a novel *APP* A673T variant was found to be associated with reduced risk of LOAD and better cognition (Jonsson et al., 2012). This variation is located near the β-secretase cleavage site and therefore makes APP a poorer substrate for β-secretase cleavage, subsequently reducing overall Aβ production. This finding provides additional proof for the hypothesis that Aβ may present the driving force of not only FAD, but also LOAD.

2.3.1.2 Common risk variants in susceptibility genes

Differening from FAD, the more common late onset form of AD (LOAD) is not caused by mutations leading to simple pathogenic outcomes, such as altered APP metabolism. Instead, LOAD is a heterogeneous disease, which results from a complex combination of various genetic risk variants and environmental factors. Most of the identified LOAD risk loci are frequent in population, but display only very small effect size. The strongest known genetic risk factor for LOAD is the ε4 variant of the apolipoprotein E (ApoE)-encoding gene (*APOE*)(Table 1)(Strittmatter et al., 1993). Approximately 50% LOAD patients have an *APOE* ε4 allele carriers. Individuals carrying one ε4 allele have four-fold increased risk for developing AD, while those who are homozygous for the ε4 allele are at 10-fold greater risk. The presence of this risk allele also decreases the age of disease onset in a dose-dependent fashion. In contrast, another variant of this gene, *APOE*, the ε2 allele (in short notation, *APOE ε2*), has been demonstrated to associate with reduced risk of AD or later age of onset (Benjamin et al., 1994). ApoE has several essential functions in the CNS and it is not entirely clear which of those are important for AD pathogenesis. A plausible mechanism might be reduced clearance and/or increased formation of Aβ in the presence of the *ε*4 genotype, but many other mechanisms have also been suggested (Bales et al., 2009, Castellano et al., 2011, Chen et al., 2010, Deane et al., 2008, Hamanaka et al., 2000, Keene et al., 2011, LaDu et al., 1994, Morris et al., 2010, Ramanan et al., 2014, Reiman et al., 2009, Verghese et al., 2013, Wolf et al., 2013). Importantly, the ApoE protein is the principal carrier of cholesterol in the brain and the ε4 isoform has the poorest cholesterol-binding capacity. Both epidemiological and experimental data strongly support the direct role of cholesterol homeostasis in Aβ accumulation and AD risk (Grimm et al., 2013, Puglielli et al., 2003). Interestingly, recent genome-wide association studies (GWAS) and next generation sequencing analysis have identified several other risk loci in or nearby genes that are functionally linked to cholesterol metabolism, transport, or uptake (**Table 1**) (www.Alzgene.org (Harold et al., 2009, Hollingworth et al., 2011, Lambert et al., 2009, Naj et al., 2011). Although the impact of these common variants on AD risk are small (10-15%), their identification has broadened the picture of the central molecular pathways involved in LOAD pathogenesis. Besides lipid

homeostasis, most of the LOAD risk genes seem to play a role in immune responce/inflammation or endosomal vesicle transport (**Table 1**) (Bertram et al., 2008, Guerreiro et al., 2013, Harold et al., 2009, Hollingworth et al., 2011, Jonsson et al., 2013, Lambert et al., 2009, Naj et al., 2011). It is noteworthy that many of these common risk variants also appear to affect Aβ levels (Chan et al., 2008, DeMattos et al., 2004, Schmidt et al., 2007, Spoelgen et al., 2006) or tau toxicity (Chapuis et al., 2013) (Shulman et al., 2014) in the brain. Taken together, identification of common LOAD risk loci and the associated molecular pathways have created whole new avenues for potential therapeutic targeting. Whether these recently implicated pathological changes take place downstream or upstream of Aβ accumulation is still under debate.

Table 1. List of AD-associated genes

Gene(s) associated with variant	**Protein**	**Pathways**	**Potential Effect on APP and tau**
ABCA7	ATP-binding cassette, sub-family A (ABC1), member 7	Lipid metabolism Immune response	Aβ clearance
AKAP9	A kinase (PRKA) anchor protein 9	Cell signaling	
ADAM10	A disintegrin and metalloprotease 10	APP processing	Cleaves APP
APP	Amyloid β (A4) precursor protein	APP processing	Cleavage releases Aβ
APOE (ε4)	Apolipoprotein E	Lipid metabolism	Aβ clearance
BIN1	Bridging integrator 1	Endocytosis Synapse function	Mediates tau toxicity
CASS4	Cas scaffolding protein family member 4	Cytoskeleton and axonal transport	
CELF1 (and *MADD)*	Elav-like family member 1	Neural development	Mediates tau toxicity
CD2AP	CD2-associated protein	Synapse function Endocytosis	Mediates tau toxicity
CD33	CD33 molecule	Immune response	Aβ clearance
CLU	Clusterin	Lipid metabolism Immune response	Aβ clearance
CR1	Complement component (3b/4b) receptor 1	Immune responce	Aβ clearance
DSG2	Desmoglein 2		
EPHA1	Ephrin type-A receptor 1	Immune response Neural development	
FERMT2	Fermitin family member 2	Cytoskeleton and axonal transport	Mediates tau toxicity
HLA-DRB5-HLA-DRB1	Major histocompatibility complex, class II, DR β5, β1	Immune response	
INPP5D	Inositol polyphosphate-5-phosphatase, 145 kDa	Immune response	
MEF2C	Myocyte enhancer factor 2C	Neural development Synapse function Immune response	

Adapted from Karch et al., 2014, Neuron 83:11-26

Table 1. Continues

Gene(s) associated with variant	Protein	Pathways	Potential Effect on APP and tau
MS4A cluster	Membrane-spanning 4-domains, subfamily A	Immune response	
NME8	NME/NM23 family member 8		
PDL3	Phospholipase D family member 3		APP trafficking and cleavage
PICALM	Phosphatidylinositol binding clathrin assembly protein	Synapse function Endocytosis	APP trafficking Aβ clearance
PSEN1	Presenilin-1	APP processing	Cleaves APP
PSEN2	Presenilin-2	APP processing	Cleaves APP
PTK2B	Protein tyrosine kinase 2 β	Synapse function Neural development	
SLC24A4	Solute carrier family 24 (sodium/potassium/calcium exchanger), member 4	Neural development Synapse function Endocytosis	
SORL1	Sortilin-related receptor, L(DLR class) A repeats containing	Lipid metabolism Synapse function Endocytosis	APP trafficking
TREM2	Triggering receptor expressed on myeloid cells 2	Immune response	Aβ clearance
ZCWPW1	Zinc finger, CW type with PWWP domain 1	Neural development	

Adapted from Karch et al., 2014, Neuron 83:11-26

2.3.2 Environmental risk factors for Alzheimer's disease: Effects of dietary lipids

Besides advanced age, female gender, genetic background, family history of dementia, depression and some other co-morbidities, there are several environmental and lifestyle factors, which often co-occur and interact across the lifespan to determine the risk of developing AD later in life (**Table 2**) (Sindi et al., 2015). Vascular and metabolic factors, including hypertension, dyslipidemia, hyperglycemia, higher BMI (both overweight and obesity), T2DM, as well as cardio- and cerebrovascular disorders have been linked to increased AD risk in epidemiologic studies. Most of these are directly linked to lifestyle factors, such as smoking, alcohol consumption, amount of physical exercise, dietary choices, and the use of antihypertensive and cholesterol lowering drugs (statins) (**Table 2**). In particular, dietary lipid composition has been implicated to be important in prevention and as a treatment strategy for cardiovascular diseases as well as T2DM and is proposed to influence AD risk and pathophysiology in epidemiological and experimental studies (Morris and Tangney, 2014). Cholesterol dyshomeostasis increases the risk of AD. On the other hand, plasma cholesterol levels are mainly affected by the saturated and polyunsaturated fatty acid (SFA and PUFA, respectively) composition in the diet. It has been demonstrated that 'typical Western-type diet' containing a considerable amount of red meat, dairy products and processed foods, rich in SFAs, trans fats and cholesterol and may increase the risk of mild cognitive impairment (MCI), dementia or AD (Barnard et al., 2014, Eskelinen et al., 2008,

Table 2. Probable and possible factors increasing and reducing the risk of AD

Risk-increasing factors	**Protective factors**
Age • >65	
Genetic • Family history • *APOE, ε4* allele • Common variants in several susceptibility genes	*Genetic* • *APP* A673T*, APOE ε2* allele • Common variants in a few susceptibility genes
Vascular and metabolic factors • Midlife hypertension • Cardiovascular diseases • Cerebrovascular lesions • Dyslipidemia/High cholesterol • Overweight or obesity • Altered peripheral glucose metabolism and insulin sensitivity, T2DM	*Lifestyle and dietary factors* • Cognitive reserve: Education, social networks, mental exercise • Physical exercise • Moderate alcohol consumption • 'Mediterranean diet' including vegetables and fish, enriched with MUFAs, PUFAs, and *n-3* fatty acids • Other dietary supplements with potentially beneficial effects: vitamins B6, B12, vitamin C,D, E, DHA, uridine, flavonoids, plant sterols, curcumin, selenium
Lifestyle and dietary factors • Smoking • Heavy alcohol use • 'Typical Western diet' rich in SFAs, trans fats and cholesterol • Increased *n-6*/*n-3* PUFA ratio	*Drugs* • Antihypertensive drugs • Statins • NSAIDs (may also have severe side-effects) • Post menopausal hormone therapy • Cholinesterase inhibitors (for treating behavioral symptoms in AD) • NMDA antagonists (for treating behavioral symptoms in AD) • GABA transporter inhibitors
Others • Female gender • History of depression • Stress • Head injury	
Others • Female gender • History of depression • Traumatic brain injury • Infectious agents	

APOE, apolipoprotein E-encoding gene; *APP* A673T, protective variant in amyloid precursor protein-encoging gene; DHA, docosahexaenoic acid; GABA, gamma-aminobutyric acid; MUFA, monounsaturated fatty acid; NMDA, N-methyl-D-aspartate; NSAID, non-steroidal anti-inflammatory drugs; PUFA, polyunsaturated fatty acid; SFA, saturated fatty acid; T2DM, type 2 diabetes mellitus.

Grimm et al., 2013, Laitinen et al., 2006, Luchsinger et al., 2002, Morris et al., 2003a). Moreover, this type of diet predisposes to weight gain and obesity, which may be independent risk factors for AD (Kivipelto et al., 2005, Mrak, 2009). On the contrary, a Mediterranean-like diet, which consists mostly of vegetables and fish, and is therefore enriched with PUFAs, has been linked to a reduced risk of cognitive decline or AD ((Barberger-Gateau et al., 2005, Eskelinen et al., 2008, Laitinen et al., 2006, Morris et al., 2003b, Tangney et al., 2014). In particular, the consumption of marine fish and other sources of *n-3* (ω-3) PUFAs, which leads to decreased *n-6*/*n-3* ratios, has been reported to lower the risk of

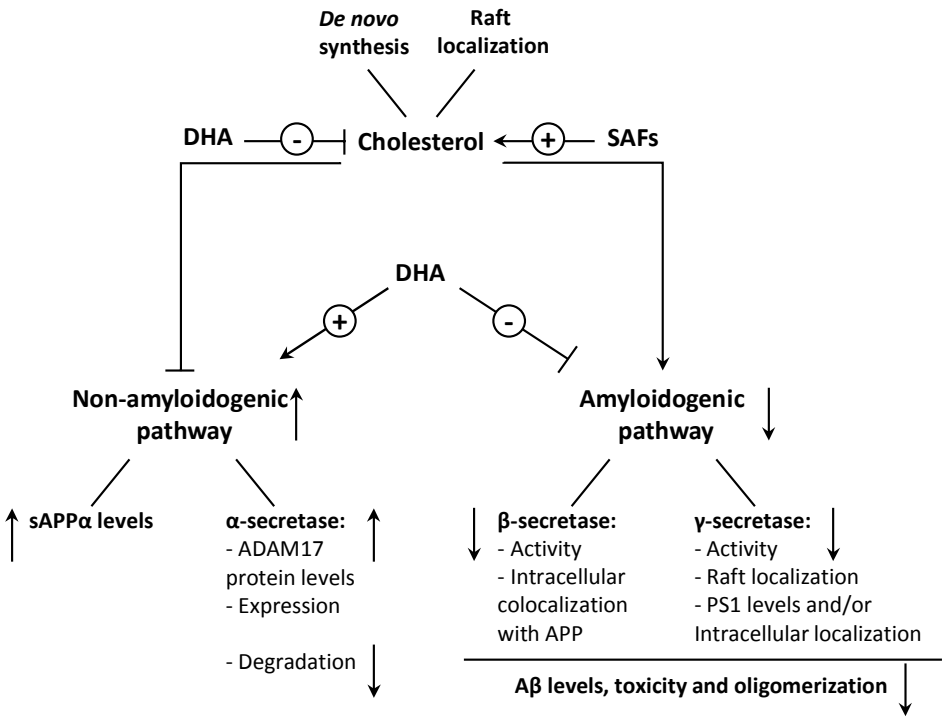

Figure 11. An illustrative summary of the possible effects of DHA on Aβ pathology. DHA decreases Aβ burden and toxicity by multiple pleiotropic effects. DHA increases non-amyloidogenic processing by increasing ADAM17 protein levels through increasing its expression and decreasing degradation. Evidence of increased sAPPα levels supports this effect. DHA also decreases amyloidogenic processing of APP by inhibiting β- and γ-secretases and altering their subcellular and/or raft localization. DHA also decreases cholesterol levels and lipid raft localization. In contrast, dietary SFAs increase the cholesterol levels. Cholesterol on the other hand increases amyloidogenic processing by increasing the activity of β- and γ-secretases and increasing protein levels of BACE1 and PS1. Cholesterol also decreases non-amyloidogenic processing of APP by acting on ADAM10. (Adapted from Grimm et al. "Docosahexaenoic acid reduces amyloid beta production via multiple pleiotropic mechanisms", *The J Biol Chem*. 2011; 286:14028-39, with the permission of the American Society for Biochemistry and Molecular Biology).

AD in an *APOE ε4-* allele dependent manner (Barberger-Gateau et al., 2005, Morris et al., 2003b, Samieri et al., 2011, Vandal et al., 2014).

Further accumulating evidence from preclinical studies also lends support these initial findings and this implies that dietary fatty acids may influence on AD-related pathology in experimental models. A high-fat diet (HFD), enriched with SFAs, is a common way to induce T2DM in rodents. T2DM and AD are risk factors for each other (Carlsson, 2010, Irie et al., 2008, Janson et al., 2004) and the diabetic phenotype (in other words peripheral hyperinsulinemia, hyperglycemia and/or insulin resistance) has been found to induce AD-type neuropathological changes in the brain of genetically and nutritionally modified rodent models of both type 1 and type 2 diabetes (Jeon et al., 2012, Jung et al., 2011, Jung et al., 2013, Kim et al., 2009a, Li et al., 2007, Moroz et al., 2008, Schubert et al., 2003). In addition, accelerated Aβ burden, tau phosphorylation and/or levels as well as cognitive decline have been observed after HFD administration in rodent models of AD (Hiltunen et al., 2012, Ho et al., 2004, Julien et al., 2010, Kohjima et al., 2010, Leboucher et al., 2013, Oksman et al., 2006, Takeda et al., 2010, Vandal et al., 2014). These HFD-induced changes have been reported to co-occur with T2DM-related peripheral changes in some, but not in all studies, implying that exacerbated AD pathology does not explicitly result from having a diabetic phenotype, but may be linked to other diet-related factors such as diet-induced obesity, dyslipidemia or aggravated neuroinflammation (Koga et al., 2014, Leboucher et al., 2013). Altogether, it is increasingly evident that dietary SFAs contribute to the development or progression of AD-type neuropathological changes, although the mechanisms by which they influence brain pathology remain partially unknown.

Other experimental data provided support for a beneficial effect of long-term dietary intake of docosahexaenoic acid (DHA), the major *n-3* PUFA. DHA intake has been shown to alleviate cognitive decline as well as brain Aβ burden and tau pathology via number of pleiotropic mechanisms in animal and cell models of AD (**Figure 11)** (Arsenault et al., 2011, Calon et al., 2004, Green et al., 2007, Grimm et al., 2011, Kariv-Inbal et al., 2012, Lim et al., 2005, Ma et al., 2007, Perez et al., 2010). More precisely, DHA has been shown to reduce Aβ oligomerization, fibrillarization and toxicity (Florent et al., 2006, Hossain et al., 2009, Teng et al., 2015, Wang et al., 2010). DHA also reduces Aβ production by both inhibiting β- and γ-secretases and by increasing the activity of the α-secretase (Grimm et al., 2011, Oksman et al., 2006, Sahlin et al., 2007, Zhao et al., 2011). It has been shown that DHA can alter lipid raft structure and mediate a restribution in the subcellular localization of APP and thus affect its co-localization with BACE1 (Grimm et al., 2011, Ma et al., 2007). Furthermore, DHA may influence AD-related pathology through affecting cerebral insulin signaling and inflammation (Ma et al., 2007, Zhao et al., 2011). In accordance to these effects, DHA depletion has been found to result in dendritic pathology and cognitive defects in AD mice (Calon et al., 2005). However, the evidence from systematic reviews suggest that DHA alone is not able to sufficiently slow down AD pathology (Lim et al., 2006). Several studies have shown that the combination of DHA and uridine monophosphate (UMP) can act synergistically in stimulating membrane lipid synthesis, increasing dendritic spine density that lead to improved learning and memory in rodents (Holguin et al., 2008, Sakamoto et al., 2007, Wurtman et al., 2006). These effects are considered to be relevant for AD pathology, which includes disturbances in membrane structure and lipid composition and subsequent loss of

synapses (van Wijk et al., 2014). In line with this, Fortasyn, which is a specific multinutrient combination designed to ameliorate synapse loss and dysfunction in AD, by providing neuronal membrane precursors and cofactors, such as UMP, the *n-3* PUFAs, DHA and eicosapentaenoic acid (EPA), choline, phospholipids, folic acid, vitamins B12, B6, C, E, and selenium, has been shown to improve cognitive performance, protect against Aβ42 pathology, enhance synaptic membrane formation and neurotransmitter signaling in rodent models of AD (Broersen et al., 2013, Cansev et al., 2015, de Wilde et al., 2011, van Wijk et al., 2014). Importantly, the same multinutrient composition, which is present in a medical food product called Souvenaid has recently been shown to preserve brain functional networks and increase memory performance in patients with mild AD (de Waal et al., 2014, Scheltens et al., 2010, Scheltens et al., 2012). Another potential group of beneficial nutrients with regard to AD neuropathology are plant sterols, which are cholesterol equivalents in plants obtained from nuts, seeds, legumes and unrefined plant oils and are widely used as cholesterol-lowering dietary agents (Malar and Devi, 2014). The beneficial properties of plant sterols may arise from their antioxidant capacity and the ability to scavenge free radicals formed during the pathological process. Taking all this evidence together suggests that the modulation of dietary composition may be a potentially practical way to influence AD risk or progression.

3 Aims of the Study

AD is the leading cause of dementia in the elderly and is listed amongst the top health issues today, by the WHO. LOAD is a heterogeneous and complex disease, which involves a poorly understood interplay between a number of genes and environmental factors. Currently, there is neither a cure nor effective disease-modifying therapies available for this disease. While the population ages, there is an urgent need to design novel prediction, prevention and intervention approaches, and to identify novel biomarker and therapeutic targets. To reach these aims, a better understanding of the molecular mechanisms underlying AD is required.

The specific aims of this thesis were:

1. To review the role of the AD risk gene *UBQLN1* and protein product in AD pathogenesis and consider its role as a potential therapeutic target in AD on the basis of recent literature (Study I).
2. To characterize the expression and functional mechanisms of the *UBQLN1* gene and protein product in AD molecular pathogenesis in the human AD brain as well as *in vitro* and *in vivo* models of AD (Study II).
3. To investigate the effect of genetic and dietary manipulation on tau-related changes in aging mice (Study III).
4. To study the molecular and behavioral effects of nutritional supplements in fish oil-based diets in the APPswe/PS1dE9 (APdE9) mouse model of AD (Study IV).

4 Materials and methods

4.1 cDNA CONSTRUCTS, siRNAs, AND VIRAL VECTORS

Different cDNA constructs containing the human *BACE1* gene that produce the protein namesake under different promoters and regulatory regions, the human full-length ubiquilin-1 transcript variant 1 (TV1) or the human wild-type 0N4R-tau were used for cell culture experiments in study II and are listed in **Table 3**. Monomeric red fluorescent protein (mRFP), enhanced farnesylated green fluorescent protein (EGFP-F), and empty plasmid DNA (pcDNA3.1) were used as controls. 5'UTR-BACE1 and 5'3'UTR-BACE1 constructs were a generous gift from Professor Stefan Lichtenthaler (DZNE, Munich, Germany). Silencer™ Pre-designed siRNA targeted to exon 5 of the human ubiquilin-1 gene was used for downregulation of ubiquilin-1 expression (Ambion). Silencer™ Negative control #1 siRNA was used as a control in RNA interference experiments (Ambion). Genes encoding human ubiquilin-1 TV1 and GFP under CAG-promoter in HIV vector were used for lentivirus-mediated gene transfer into embryonic mouse primary neurons and hippocampi of adult APdE9 mice (**Table 3**). All lentiviral constructs were ordered from the National Virus Vector Laboratory, A.I. Virtanen Institute, University of Eastern Finland. Lentiviral particles containing empty HIV-plasmid with the CAG-promoter, but lacking the coding insert were used as a transduction control.

Table 3. cDNA constructs, siRNAs and lentiviral vectors

cDNA construct	Description	Supplier or original reference
5'3'UTR-BACE1	Human BACE1 containing endogenous promoter and 5'- and 3'UTR regulatory regions	Prof. Stefan Lichtenthaler
5'UTR-BACE1	Human BACE1 containing endogenous promoter and 5'UTR regulatory region	Prof. Stefan Lichtenthaler
BACE1-myc	Human BACE1 under CMV promoter and containing 3' myc-tag	
TV1	TV1 of human ubiquilin-1	(Lu et al., 2009)
TV1-myc	TV1 of human ubiquilin-1 containing 5'myc tag	(Viswanathan et al., 2011)
myc-TV1-mRFP	TV1 of human ubiquilin-1 containing 5'myc and 3'mRFP tag	(Viswanathan et al., 2011)
0N4R-tau	Human wild type 0N4R-tau	GeneCopoeia
mRFP	Monomeric red fluorescent protein	
EGFP-F	Enhanced green fluorescent protein, farnesylated	Clontech
pcDNA3.1	Empty plasmid cDNA	(Lu et al., 2009)
UBQLN1 siRNA	Silencer™ Pre-designed siRNA targeted to exon 5 of the human ubiquilin-1 gene (GGCGCATGTACACAGATAT)	Ambion
Control siRNA	Silencer™ Negative control #1 siRNA	Ambion
TV1-HIV-pBOB	TV1 of human ubiquilin-1 in lentiviral vector under CAG-promoter	National Virus Vector Laboratory, A.I. Virtanen Institute, University of Eastern Finland
GFP-HIV-pBOB	Green fluorescent protein in lentiviral vector under CAG-promoter	National Virus Vector Laboratory, A.I. Virtanen Institute, University of Eastern Finland
HIV-pBOB-CAG	Empty lentiviral vector and CAG-promoter without coding insert	National Virus Vector Laboratory, A.I. Virtanen Institute, University of Eastern Finland

4.2 CELL CULTURE EXPERIMENTS

4.2.1 Cell lines and transfections

Secondary cell lines used in study II are listed in **Table 4**. The HEK293-AP-APP cell line was a kind gift from prof. Stefan Lichtenthaler, DZNE, Munich, Germany (**Table 4**). Cells were cultured in a humidified cell culture incubator in 5% CO_2 atmosphere in Dulbecco's Modified Eagle's Medium (DMEM), supplemented with 10% fetal bovine serum (FBS), 2 mM L-glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin and appropriate selective antibiotic

(**Table 4**). For HEK293-AP-APP cells, culture plates were coated with poly-D-lysine (100 µg/ml, PDL; Sigma) before transfections. All transfections were performed using Lipofectamine 2000-reagent (Invitrogen) in Opti-MEM® I –Reduced serum medium (Gibco) according to manufacturer's instructions. Medium was replaced 4 or 24 h later and proteins were extracted 24 or 48 h after transfections, respectively. Protein samples were analyzed with Western blot.

Table 4. Secondary cell lines

cell line	Description	Selection antibiotic	Reference
SH-SY5Y-APP751	Human neuroblastoma cells stably overexpressing APP751 isoform	200 µg/ml geneticin (G418, Gibco)	(Sarajarvi et al., 2009)
H4-pcDNA-A	Human neuroglioma cells stably overexpressing pcDNA	200 µg/ml geneticin (G418, Gibco)	(Lu et al., 2009)
H4-APP751	Human neuroglioma cells stably overexpressing APP751 isoform	200 µg/ml geneticin (G418, Gibco)	(Hiltunen et al., 2006)
H4-TV1 (clone-13)	Human neuroglioma cells stably overexpressing TV1 of human ubiquilin-1 (clone 13)	200 µg/ml geneticin (G418, Gibco)	(Lu et al., 2009)
HEK293-AP-APP	Human embryonic kidney cells stably overexpressing APP695 isoform linked to alkaline phosphatase	0.3µg/ml puromycin and 50µg/ml hygromycin B (Invitrogen)	(Lichtenthaler et al., 2003)
BV2	Mouse microglial cells		(Gresa-Arribas et al., 2012)

4.2.2 Cycloheximide time course assay

Twenty-four hours after transfection, H4 pcDNA-A and H4 TV1-13 cells transfected with 5'3'UTR-BACE1 were treated with 30 µg/ml of cycloheximide to halt protein biosynthesis. To assess protein stability, proteins were extracted at 0, 6, and 12 h time points, subjected to Western blot and analyzed with BACE1-specific antibody (PA1-757; 1:1000). The half-life of BACE1 was calculated by quantifying BACE1 protein levels with respect to different treatment times. BACE1 protein intensities in Western blots at different time points were normalized to BACE1 levels at 0 h.

4.2.3 Immunofluorescense and microscopy

SH-SY5Y-APP751 cells were plated on PDL-coated glass coverslips and transfected with 5'3'UTR-BACE and myc-TV1 or 5'3'UTR-BACE and pcDNA3.1, to assess subcellular localization with fluorescent microscopy. Twenty-four h after transfections, cells were fixed with 4% PFA for 15 min at room temperature. Next, the cells were permeabilized in PBS containing 0.1% Triton-X100 (Sigma) for 10 min. Non-specific antibody staining was blocked with PBS containing 1.5% goat IgG (Zymed) for 30 min. Cells were then incubated for 1.5 h at room temperature with following primary antibodies (**Table 7)**: BACE1 (D10E5; 1:250), myc (1:100), Rab7 (1:50), TfR (1:200), EEA1 (1:100). This was followed by incubation with Alexa Fluor488 goat anti-rabbit (1:500; Molecular Probes) and Alexa Fluor594 goat anti-mouse (1:500; Molecular Probes) secondary antibodies for 1 h. Coverslips were mounted with

Vectashield mounting media containing DAPI (Vector Labs). The stainined cells were then imaged with a Zeiss Axio Imager using ApoTome.2 at 63 x magnification. Images were then analyzed and quantified with Zeiss ZEN Module Colocalization analysis hardware.

4.2.4 Preparation of co-cultures of embryonic mouse primary cortical neurons with BV2 microglial cells, lentivirus-mediated gene transductions, and experimentally induced neuroinflammation

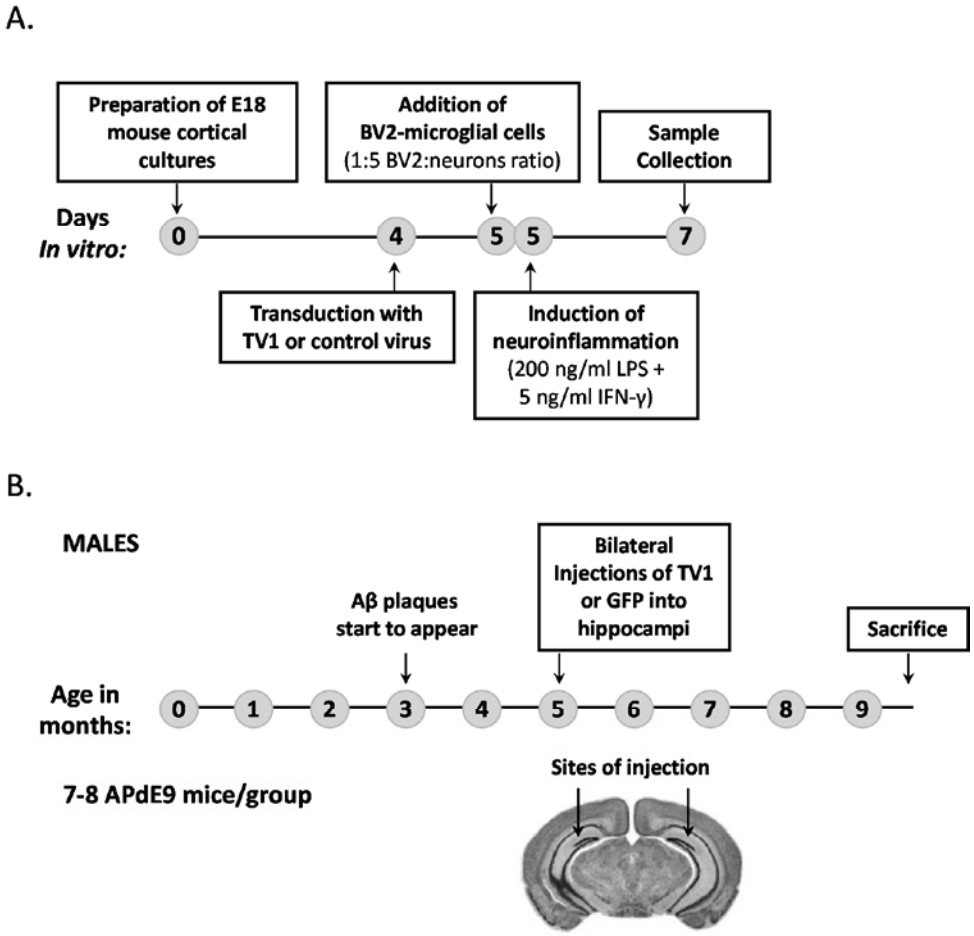

Figure 12. Experimental design of study II. A) Illustration of lentivirus-mediated transduction of ubiquilin-1 TV1 or GFP in embryonic mouse primary cortical neurons on fourth day *in vitro* (DIV4). On DIV5, the viral particles were removed and mouse microglial BV2 cells were added on neuronal cultures on 1:5 ration (BV2:neurons). Two hours later, neuroinflammation was induced with 200 ng/ml of lipopolysaccharide (LPS) and 5 ng/ml of interferon-γ (IFN-γ). Samples were collected for biochemical analyses on the seventh day. B) A schematic presentation of lentivirus-mediated bilateral injections of TV1 or GFP into the hippocampi of five month old male APdE9 mice. Aβ plaques start to appear at the age of 3 months. The mice were sacrificed at the age of 9.5 months.

JAXC57BL/6J female mice were euthanized by cervical dislocation at the 18th day of the pregnancy. Pups at the embryonic day 18 (E18) were sacrificed and their cortices were

dissected in DMEM containing 10% FBS (dissection medium), under the microscope. The isolated cortices were then rinsed with HC-buffer [Phosphate buffered saline, PBS, supplemented with 1 mg/ml bovine serum albumin (BSA) and 10 mM glucose] and extracellular matrices were proteolytically-digested with 0.25% trypsin in DMEM for 20 min at +37°C to yield a suspension of single cells. The trypsin reaction was terminated with an equal amount of DMEM containing 10% FBS, 100 U/ml penicillin and 100 μg/ml streptomycin (plating medium). Cell suspensions were then centrifuged at 200 *x g* for 5 min. The resultant pellets containing the cells were re-suspended by triturating ~20 times in plating medium and filtered through a 40 μM diameter mesh cell strainer. The cells were further centrifuged at 150 *x g* for 5min, then re-suspended into Neurobasal-medium (Gibco), supplemented with B27 (Gibco), 2 mM L-glutamine and 100 U/ml penicillin and 100 μg/ml streptomycin. Finally cell numbers were counted with a hemocytometer and plated on 48- or 24-well plates at the density of 200 000 or 345 000 cells/well, respectively.

On the fourth day *in vitro*, primary cortical neurons were transduced with lentiviral particles encoding human *UBQLN1* TV1, GFP, or CAG-promoter without coding insert (**Figure 12A**). Lentiviral particles were adjusted to a 25 MOI (multiplicity of infection) with PBS. Next day, the medium containing lentiviruses was replaced. BV2 coculture cells were carefully scraped in Neurobasal-BV2 medium, counted with hemocytometer and added at one-to-five ratio (BV2 to neurons) on the neuronal cultures (Gresa-Arribas et al., 2012). Two hours later, neuroinflammation was induced with the combined treatment 200 ng/ml of lipopolysaccharide (LPS; Sigma) and 5 ng/ml of interferon-γ (IFN-γ; Sigma). Samples were analyzed 48 h later. Evaluation of GFP-transduced samples under a fluorescent microscope revealed a transduction efficacy of lentiviral particles of approximately 60%.

4.3 HUMAN BRAIN SAMPLES

The human neuropathological sample cohort analyzed in study II included temporal cortex samples from sixty individuals (18 males and 42 females, mean age 80.6 ± 8.9) who were examined at the Memory Clinic of the Department of Neurology, Kuopio University Hospital (**Table 5**). Among these individuals, 41 were diagnosed as probable AD according to the National Institute of Neurological and Communicative Disorders and Stroke and the Alzheimer's Disease and Related Disorders Association (NINCDS-ADRDA) criteria (McKhann et al., 1984). After death, the subjects were autopsied and evaluated for AD-related neurofibrillary pathology with immunostaining of paraffin sections with AT8 antibody, which detects hyperphosphorylated tau (Braak et al., 2006). The subjects were classified based on the degree of neurofibrillary pathology and divided in three groups according to Braak staging: Braak stage 0-II (n=28), Braak stage III-IV (n=13), and Braak stage V-VI (n=19) (Natunen et al., 2013a, Natunen et al., 2013b). Fresh frozen tissue samples from the same cases were used for RNA and protein analysis. The Ethics Committee of the Kuopio University Hospital, The Finnish National Supervisory Authority, and The Finnish Ministry of Social Affairs and Health approved the study.

Table 5. Demographics of Neuropathological sample cohort

	Neuropathological sample cohort (n=60)		
	Braak 0-II (n=28)	Braak III-IV (n=13)	Braak V-VI (n=19)
Clinical Status: control/demented	17/11	2/11	0/19
Age at death, mean ± SD, (y)	79.2 ± 10.9	82.9 ± 6.8	81.3 ± 7.0
Gender: males/females	14/14	2/11	2/17
PMD, mean ± SD (h)	18.0 ± 19.6	15.8 ± 16.1	7.1 ± 5.0
Brain weight (g)	1250 ± 183	1104 ±156	1040 ± 183

4.4 ANIMAL EXPERIMENTS

4.4.1 Animals

Transgenic APdE9 mice were used as a model for AD in studies II, III and IV. APdE9 mice overexpress chimeric mouse/human APPswe (mouse *App695* harboring a humanised Aβ domain in conjunction with the "Swedish" familial mutations K959N and M596L) as well as the human *PS1-ΔE9* (deletion of exon 9) gene. Both genes epression being controlled by independent mouse prion protein promoter elements (Jankowsky et al., 2004). In these mice, the formation of amyloid plaques in the cortex and hippocampus starts at the age of three months, proceeding faster in females. Transgenic mice overexpressing the mouse insulin-like growth factor 2 (*IGF2*) gene and protein product exclusively in the pancreas were used as a model for T2DM in the study III (Devedjian et al., 2000). In these mice, the pancreatic β-islet cell hyperplasia leads to insulin overproduction and a subsequent development of resistance to this hormone (Hiltunen et al., 2012). For the study III, these two mouse lines were crossbred to create a triple transgenic APdE9xIGF2 mouse line. All mouse lines were backcrossed into the common C57BL6/J line for at least 10 generations. Age-matched wild-type littermates were used as controls in studies III and IV. Mice were housed in communal group cages until behavioral testing began, in a controlled environment (temperature +22˚C, humidity 50-60%, lights period 07:00-19:00 h) with fresh food and water available, *ad libitum.* All animal procedures were carried out according to the European Community Council Directives (Directive 86/609/EEC) and Finnish guidelines, and approved by the Animal Experiment Board of Finland.

4.4.2 Hippocampal injections of lentiviral vectors

The study design of lentiviral injections conducted in studuy II is shown in the **Figure 12B**. Stereotactic injections of lentiviral particles into five-month-old APdE9 male mice hippocampi were conducted by Prof. Heikki Tanila's group at the A.I.V. Institute of University of Eastern Finland. Briefly, lentiviral vectors containing *UBQLN1* TV1 and GFP genes were adjusted to a titer of 1.28×10^9 in PBS. Mice were anesthetized with halothane and placed in a stereotactic frame (model 900, David Kopf). *UBQLN1* TV1 (n=8) and GFP (n=8) containing lentiviral preparations in 2 µl volume were bilaterally injected to the dentate gyrus of the hippocampus by using the following coordinates: ± 3.2 mm lateral, 2.7 mm

posterior, and 2.7 mm ventral (Bregma coordinates). The viral particles were injected at a speed of 0.2 μl/min by using a Hamilton 10 μl syringe and 28 G needle. Sham-operated mice were used as controls. Post-operatively, the mice received 5 mg/kg of carprofene (Rimadyl 50 mg/ml) sub-cutaneously (s.c.) and were allowed to recover in a heated chamber. The animals were then sacrificed at the age of 9.5 months (**Figure 12B**). Lentiviral particles were estimated to infect ~20% of neurons of the dentate gyrus of the hippocampus, as judged by immunohistochemical (IHC) analysis of postmorten fixed brain slices with antibody specifically detecting human origing ubiquilin-1 (see Chapter 4.5).

4.4.3 Diets and experimental designs

The detailed composition of the experimental diets used in studies III and IV are listed in **Table 6** and the course of each experiment is shown in **Figures 13A** and **B**. In study III, the combined effect of genetic and dietary manipulation was investigated, in both female and male mice, with four different genetic backgrounds: wild-type (AwIw), *APdE9* transgenic (A+Iw), *IGF2* transgenic (AwI+), and *APdE9*x*IGF2* triple transgenic (A+I+) mice (**Figure 13A)** (Hiltunen et al., 2012). Beginning at the age of five months (females) or eight months (males), the mice were fed either with standard laboratory chow, containing 5% (w) fat and no cholesterol (standard diet, STD; R36; Lactamin, Sweden) or with a chow mimicking typical Western-type high-fat diet (HFD) containing 21% (w) fat and 0.2% cholesterol (**Table 6**; n=4-6 mice/group) (adjusted calories diet; TD 88137; Harlan Tekland, USA). Female mice underwent a behavioral test battery at the age of 8 months and were sacrificed at nine months of age (Hiltunen et al., 2012). Male mice went through the same behavioral test battery during the two last weeks before sacrifice at the age of 12 months (**Figure 13A**). The weight gain of all mice was monitored throughout the experiment. In addition, the female mice underwent glucose and insulin tolerance tests (GTT and ITT, respectively) to assess peripheral metabolic changes during experimental diet exposure. These tests were performed, and described in more detail, in a previous study and used only for correlation analyses in study III (Hiltunen et al., 2012).

Table 6. Specific percentage composition of experimental diets

	Control	FO	Fortasyn	(FOPS)	TWD
Carbohydrates					
Corn starch	35.57	35.57	32.78	35.32	15.00
Casein	14.00	14.00	14.00	14.00	19.50
Corn dextrin	15.50	15.50	15.50	15.50	
Sucrose	10.00	10.00	10.00	10.00	34.15
Dextrose	10.00	10.00	10.00	10.00	
Cellulose					5.00
Fibers	5.00	5.00	5.00	5.00	
Mineral mix (AIN-93M-MX)	3.50	3.50	3.50	3.50	
Mineral mix (AIN-76, 170915)					3.50
Vitamin mix (AIN-93-VX)	1.00	1.00	1.00	1.00	1.00
Vitamin mix (Tecland 40060)					
Fats					
Soy oil	1.90				
Coconut oil	0.90	0.10	0.10	0.10	
Corn oil	2.20	1.87	1.87	1.87	
Fish oil		3.03	3.03	3.03	
SFA (g/100 g fat)	26.54	23.00	23.00	23.00	61.80
MUFA (g/100 g fat)	21.09	22.51	22.51	22.51	27.05
PUFA (g/100 g fat)	45.77	44.11	44.11	44.11	4.80
Ratio *n6/n3*	20.30	0.98	0.98	0.98	increased
Additions					
L-Cysteine	0.18	0.18	0.18	0.18	
Choline bitartrate (41.1% choline)	0.25	0.25	0.25	0.25	
Tert-butylhydroquinone	0.0008	000.8	0.0008	0.0008	
Pyridoxine-HCl			0.0033		
Folic acid (90%)			0.0007		
Cyanocobalamin (0.1% in mannitol)			0.0035		
Ascorbic acid (100% pure)			0.16		
$_{D}$-α-Tocopheryl acetate (500 IU/g)			0.47		
UMP disodium (24% H_2O)			1.00		
Choline chloride (74.6%)			0.40		
Soy lectin			0.76		
Sodium selenite (46% min)			0.0002		
Stigmasterol (95%)				0.25	
Cholesterol (added)					0.15
Cholesterol (from fat source)					0.05
DL-methionine					0.30
Calcium carbonate					0.40
Andydrous milk fat					0.004
Ethoxyquin					21.00
Total	100.0	100.0	100.0	100.0	100.0
Energy (kcal/100 g)	376.9	376.9	365.7	375.9	450.0

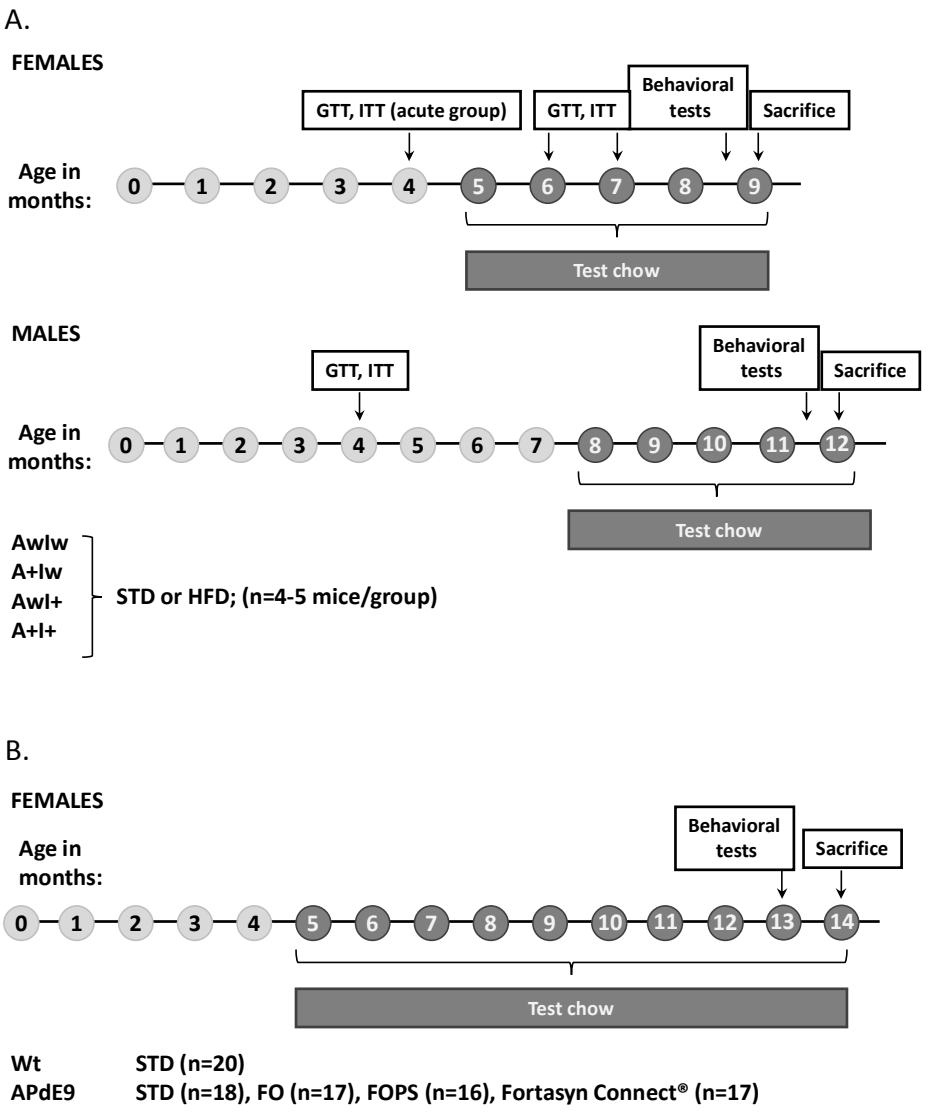

Figure 13. Experimental designs of studies III and IV. A) The female mice in study III were fed with high-fat diet (HFD) or standard diet (STD) for four months, starting at the age of five months. Glucose tolerance (GTT) and insulin tolerance (ITT) tests were performed at the age of six and seven months, respectively. Additional acute group went through GTT and ITT at the age of four months. Before sacrifice at the age of nine months, the mice went through behavioral test battery. Male mice started to receive HFD at the age of 8 months and the diet lasted for four months. The experimental diets were fed to mice with four different genetic backgrounds. AwIw indicates the wild-type mice, A+Iw indicates the APdE9 mice, AwI+ mice overexpress IGF2 in the pancreas and are the model for type 2 diabetes and A+I+ mice have all three transgenes. B) In the study IV, the female APdE9 mice were fed with special lipid-based diets including fish oil (FO), fish oil and plant sterols (FOPS) or fish oil and selected nutritional composition called Fortasyn. APdE9 mice and their wild-type littermates fed with control chow (STD) were used as a control group. Mice were fed with experimental diets for nine months, beginning at the age of five months. Before sacrificing at the age of 14 months, the mice went through a behavioral test battery.

Experimental diets used in the study IV were isocalorific and differed regarding to their fatty acid composition and other specific nutrients (**Table 6**) (all diets based on the standard AIN-93M) (Reeves et al., 1993). Starting at the age of 5 months, APdE9 female mice were fed with diets containing fish oil (FO, n=17), fish oil and plant sterols (FOPS, n=16) or fish oil and specific nutrient combination providing neuronal membrane precursors and cofactors [Fortasyn (n=17)], for 10 months (**Figure 13B**). APdE9 (n=18) and wild-type (n=20) mice fed with normal laboratory mouse chow (control) were used as controls. All mice went through a behavioral test battery at the age of 13 months and were sacrificed at the age of 14 months. To monitor normal growth, the body weight was measured first weekly and then monthly.

4.4.4 Behavioral tests

The behavioral tests mentioned in study III were conducted previously and described in detail in Hiltunen et al., 2012 (Hiltunen et al., 2012). The behavioral data obtained from this study was only used for correlation analysis in the study III. In the study IV, the 12 month-old mice underwent a 3-week neurological test battery, including the following tests in the described order. A week before the behavioral test sessions, the mice were moved to to individual cages. All tests were performed during the working hours (between 8 am and 4 pm).

4.4.4.1 Spontaneous explorative activity

Locomotor activity and spontaneous exploratory behavior was monitored in a transparent test cage (length 26 cm, width 26 cm, height 39 cm) fitted with automated infrared photodetection enabling separate monitoring of horizontal (ambulatory distance) and vertical exploratory activity (rearing) (TruScan®, Coulbourn Instruments, CO, USA). Recordings were made in two separate sessions, of a 10 minute time duration each, with a 48 h retention interval. The test cage was cleaned with 70 % EtOH between each animal to remove odor traces.

4.4.4.2 Marble burying

Marble burying task to study fear of new objects was conducted in the mouse home cage. In the afternoon, 1 liter of extra bedding was added on the bottom of the cage and nine glass marbles (1 cm in diameter) were arranged on it. Next morning (16-18 h later), the number of visible glass marbles was counted.

4.4.4.3 Odor recognition

This task was used to test olfactory recognition ability of mice and based on specific odors of individual mice. Before the test, each mouse was allowed to get used to the presence of two small odorless wooden balls (Step Systems, Lahti, Finland) in its home cage overnight. For the odor recognition test, each mouse was presented with two wooden balls, one impregnated with its own odor and one ball from the cage of the selected odor donor mouse. During the 2 min test session, total time spent sniffing the balls and the number of visits (the nose pointing to the ball at a distance < 2 cm) to each ball was recorded.

4.4.4.4 The Morris swim navigation (water maze) task

The Morris swim navigation task was used to measure spatial learning and memory. Before the test, mice were familiarized with the water (20±0.5°C) environment and allowed to learn how to find the hidden platform. The test was performed in a black plastic pool, with a diameter of 120 cm. During the first four test days, mice were put through five 60-second training trials per day with hidden escape platform placed at a constant location, and a starting point varying between four different locations at the pool rim (South, West, North, East). A 5 to 10 min recovery period was allowed between the training trials. If the mouse failed to find the hidden platform within the 60 s, it was placed on it for 10 s before moving on to the next animal. To assess the search bias, a 40-s probe trial on the fifth day was performed without the platform and the time spent near the former platform location was measured. The escape latency, thigmotaxis (wall-swimming tendency), swim path, and swimming speed were recorded on each test day with an image analyzer (HVS, Image®, Hampton, UK).

4.4.5 Brain dissections and sample preparation

At the end of the experiments, animals were deeply anesthetized with pentobarbital/chloralhydrate cocktail (60 mg/kg each) and transcardially perfused with 50 ml of heparinized 0.9% ice-cold saline (NaCl) (Studies III and IV). One hemisphere was fixed with in a 4% paraformaldehyde (PFA) containing solution for 4 h before being transferred to and incubated in a 30% sucrose solution overnight at +4°C. This hemisphere was then stored in cryoprotectant at -20˚C for later use in histochemical analyses. The other hemisphere was dissected on ice into cerebellar, hippocampal, and cortical (frontal, parietal and temporal cortex) blocks. In addition, in study IV, 37 mice were euthanized by cervical dislocation without perfusion. In this case, the brain was removed and rinsed three times in ice-cold saline. The whole left cortex was dissected as one block, while the right hemisphere was dissected to above described blocks. Dissected brain blocks were snap frozen in liquid nitrogen and stored at -70˚C for biochemical assays. Later, tissue blocks were weighed and mechanically homogenized in ice-cold PBS (10 volumes of PBS/mg tissue) or the appropriate analysis specific buffer. The homogenate in PBS was divided into two protein fractions (one with 1:100 EDTA-free protease and HALT™ phosphatase inhibitor cocktails, and one without), and a RNA fraction. In addition, the liver was collected and analyzed in studies III and IV.

4.5 IMMUNOHISTOCHEMISTRY

Immunohistochemical (IHC) analysis of mouse brain sections was conducted by Prof. Heikki Tanila's group at the A.I.V. Institute of University of Eastern Finland. Shortly, one PFA-fixed hemisphere was cut into 35 μm sections with an interval of 200 μm. The sections were pre-treated with +80˚C 0.05 M sodium citrate solution (pH 6.0) for 30 min before primary antibody incubations. The antibodies used for the histology experiments are listed in **Table 7**. Histological sections were incubated with primary antibodies detecting human Aβ (pan-Aβ, 1:2000; W02, 1:1000), BACE1 (D10E5, 1:1000), activated micrglia (CD45, 1:2000) and human ubiquilin-1 (U7258, 1:1000), at room temperature for 18 h on a shaker. Subsequently, the sections were rinsed three times with Tris-buffered saline-Tween 20 (TBS-T) and

incubated with the appropriate anti primary host antibody secondary antibody (biotinylated goat anti-mouse or goat anti-rabbit, Vector Laboratories 1:500; AlexaFluor488 goat anti-rabbit, 1:250; anti-mouse IgG CY3 conjugated 1:400, Sigma-Aldrich) for 2 h at room temperature on a shaker. Sections were rinsed three times with TBS-T, and placed into StreptAvidin (GE Healthcare, 1:000) solution. After rinsing with TBS-T, sections were incubated with Nickel-enhanced DAB solution for 3 min. Detection was made with Olympus BX40 microscope and DP50 camera, with image processing and analysis performed with the Photoshop CS3 program. The final values were calculated from grayscale images by dividing the selected area of interest with total hippocampal area.

Table 7. Primary antibodies

Antibody and target epitope	**Source and clonality**	**Cat. no**	**Dilutions**			**Supplier**
			WB[a]	**IF**[b]	**IHC**[c]	
$A\beta_{1-16}$ (6E10)	ms,M	SIG39320	1:1000			BioSite
Aβ (W02)	ms,M	MABN10			1:1000	Millipore
Akt	rbt,P		1:1000			Cell Signaling Tech.
APP, CTF	rbt,P	A8717	1:2000			Sigma
ARF6 (3A-1)	ms,M	sc-7971	1:1000			Santa Cruz
BACE1	rbt, P	PA1-757	1:1000			Pierce
BACE1 (61-3E7)	ms,M	sc-33711	1:100			Santa Cruz
BACE1 (D10E5)	rbt,M	5606	1:1000	1:250	1:1000	Cell Signaling Tech.
CD45		1388			1:2000	Serotec
DAPI				1:5000		Sigma
DHCR24/ seladin-1	rbt,P	2033	1:1000			Cell Signaling Tech.
EEA1		610456		1:100		BD Transduction Labratories
eIF2α	rbt,P	9722	1:1000			Cell Signaling Tech.
GAPDH (6C5)	ms,M	ab8245	1: 15000			Abcam
GFAP (C-terminus)	ms,M	G3893	1:1000			Sigma
GFP	ms,M	118144600	1:1000			Roche
GGA1	rbt,P	ab38454	1:100			Abcam
GGA3	ms,M	612310	1:2500			BD Transduction Labs
GSK3β	rbt,P		1:1000			Cell Signaling Tech
MAP2	m,M	M9942			1:2000	Vector Labs
myc, clone 4A6	ms,M	05-724	1:1000	1:200-1:100		Millipore
Pan-Aβ		44-136			1:2000	BioSource
p-Akt, Ser473	rbt,P					Cell Signaling Tech.

a) *Western blot; b) Immunofluorescence; c) Immunohistochemistry*
Ms, mouse; Rbt, rabbit; M, monoclonal; P, polyclonal

Table 7. Continues

Antibody and target epitope	Source and clonality	Cat. no	Dilutions			Supplier
			WB[a]	IF[b]	IHC[c]	
p-eIF2α, Ser51	rbt,P	3597	1:1000			Cell Signaling Tech.
p-GSK3β, Ser9	rbt,P		1:1000			Cell Signaling Tech
p-tau (AT8), p-Ser199, Ser202/Thr205	ms,M		1:1000			INNX
Rab-7 (B-3)		sc-376362		1:50		Santa Cruz
RD3 isoforms	ms,M		1:1000			Millipore
RD4 isoforms	ms,M		1:1000			Millipore
Tau C-17	goat anti-rbt,P		1:200			Santa Cruz
TfR	ms,M	13-6800	1:1000	1:200		Zymed
Ubiquilin-1	rbt,P	U7258	1:500-1:1000	1:1000	1:1000	Sigma
Ubiquilin-1	rbt,P		1:500			Abcam
Ubiquitin, Lys63	rbt,P	05-1308	1:1000			Millipore
Ubiquitin, Lys48	rbt,P	05-1307	1:1000			Millipore
β-actin	ms,M	Ab8226	1:1000			Abcam
β-tubulin	ms,M	MAB3408	1:2000			Millipore

a) Western blot; b) Immunofluorescence; c) Immunohistochemistry
Ms, mouse; Rbt, rabbit; M, monoclonal; P, polyclonal

4.6 BIOCHEMICAL ASSAYS

4.6.1 Custom exon chip analysis of human brain tissue samples

The RNA samples used in study II for custom exon chip analysis were extracted and validated previously (Natunen et al., 2013a). Briefly, RNA was from temporal cortex samples was performed previously by using a phenol-chloroform extraction method (21 samples) or RNeasy Lipid Tissue Mini Kit (39 samples; Qiagen) (Natunen et al., 2013a). The quality of RNA was determined with 2100 Bioanalyser (Agilent) and categorized according to the RNA integrity number (RIN) (Martiskainen et al., 2015, Natunen et al., 2013a). The distribution of RNA samples based on the RIN values was 15.1% (RIN<4), 41.5% (RIN<6), and 43.4% (RIN<8). The *post mortem* delay did not correlate with RIN values (Pearson correlation, r=-0.09, p=0.40) and the RNA quality was not associated with the expression of *UBQLN1*.

The expression microarray was performed by our collaborators at the Finnish Microarray and Sequencing Centre in Turku, Finland. Briefly, the global expression of *UBQLN1* analyzed with Agilent One-Color Microarray-Based exon Analysis by using five probes individually targeted for *UBQLN1* exons 2, 4, 6, 9, and 11. One hundred nanograms of RNA was amplified and labeled with Cy3 using Low Input Quick Amp WT Labeling kit (Agilent, product number 5190-2943). Samples were processed with the RNA Spike in kit (Agilent, product number 5188-5282) and concentration of starting material and labeled complementary RNA (cRNA) was measured by using NanoDrop ND-2000. The RNA and cRNA quality was determined with Bioanalyzer and a RNA 6000 Nano kit (Agilent, product number 5067-1511). Cy3-labeled cRNA (600 ng) was hybridized to 8x60K custom exon chip (Agilent, Design ID 044312) for overnight at +65°C using Gene Expression Hybridization kit

(Agilent, Product number 5188-5242). The custom probes used in the analysis were designed and validated with Agilent's eArray application (Martiskainen et al., 2015). The chips were washed with Gene Expression Wash Pack (Agilent Product number 5188-5327) washing buffer and scanned with Agilent Technologies Scanner (model G2565CA). The numerical data were obtained with Agilent Feature Extraction software (version 10.7.3). The expression levels of *UBQLN1* exons were normalized to the global expression of β-actin gene (*ACTBL2*). The *ACTBL2*-normalized UBQLN1 expression in Braak stage 0-II was set to 100%.

4.6.2 mRNA analysis of mouse brain tissue samples

In study III, RNA was extracted from mouse temporal cortex homogenates (in PBS) using TRIzol®-Reagent (Invitrogen). One millilitre of TRIzol® was added per 50-100 mg of tissue homogenate and incubated at room temperature for 5 min. Chloroform was then added in a one-to-five ratio to tissue homogenate and tubes were shaken vigorously. The mixture was further incubated at room temperature for 5 min and separated by centrifugation at 12000 *x g* for 15 min at +4°C. The RNA was precipitated from the aqueous phase using 0.5 ml 2-propanol per 1 ml of TRIzol®at room temperature for 10 min. The samples were then centrifuged at 12000 *x g* for 10 min at +4°C and washed twice with 75% ethanol. RNA pellets were then air-dried, dissolved in RNase-free water and stored at -80°C until needed or used directly.

Complementary DNA was synthesized from equal amount of total RNA each sample (200ng) in a reaction mixture including 10 mM dNTP, 1µl random hexamers, 4µl 1x First Strand Buffer, 0.1 M DTT, 40 U/µl Rnase out and 10000 U SuperScript™ III reverse transcriptase (Invitrogen) in a total volume of 11 µl. One µl of cDNA was used as a template for 6FAM-fluorescent labeled forward primer (10 pmol/µl) and unlabeled reverse primer (10 pmol/µl; tau 4R/3R, **Table 8**) (Suh et al., 2010) in a master mix containing 1µl 1x Buffer, 15 mM $MgCl_2$, 10 mM dNTP, 5 U/µl Hot Star Tag (Qiagen) in 10 µl final volume. Samples were PCR-amplified in two separate runs; 31 PCR cycles were used to amplify 3R-tau and 23 cycles were used to amplify 4R-tau at the 58°C annealing temperature. 3R-tau- and 4R-tau-specific PCR products were separated with capillary electrophoresis by using a POP-6 denaturing polymerase and 3100 Genetic Analyzer apparatus (Applied Biosystems). The peak heights were determined with GeneScan analysis program (Applied Biosystems) and normalized to the levels of glyceraldehyde 3-phosphatedehydrogenase (*Gapdh*) from the same samples. To validate the specificity of PCR products, 3R-tau and 4R-tau fragments were detected with agarose gel electrophoresis (1.5%). Subsequently, fragments were extracted from agarose gel using QIAquick Gel Extraction kit (Qiagen). Extracted fragments were re-amplified with tau-specific primers flanking exon 10 (tau-Seq, **Table 8**) and sequenced with 3100 Genetic Analyzer. The data was then analyzed with the Sequence Analyzer program (Applied Biosystems). RNA extracted from the cortex of E18 mouse was used as a positive control for 3R-tau fragment in the analysis. Specific primers were designed to analyze the levels of housekeeping gene *Gapdh*, serine/arginine-rich splicing factors, *Srsf10*/*Tra2*β, *Srsf9* (SRp30c), *Srsf2* (SC35), *Srsf1* (SF2/ASF), and inflammatory cytokines, tumor necrosis factor α (*Tnfα*) and interleukin 1β (*Il1β*) (**Table 8**) with SYBR Green Master PCR Mix (Applied Biosystems) and real-time quantitative PCR (7500 Fast Real Time PCR System, Applied Biosystems). All target mRNA levels were normalized to those of *Gapdh* from the same samples.

Table 8. Primers and target sequences

Target	**Primer sequence 5'-3'**	
	Forward	Reverse
MAPT 4R/3R	FAM6-TAAGACTCCTCCAGGGTCAG	ACTTGGAGGTCACTTTGCTC
MAPT-Seq	TAAGTCACCATCAGCTAGTA	AGGTCACTTTGCTCAGGTCC
Tra2β	TACTCGAAGGCGTCATGTTG	ATTCCATTGGCACGTTCTTT
***Srsf9* (Srp30c)**	TGCTCTGCGTAAACTGGATG	AGGGCCTGAAAGGAGAGAAG
***Srsf2* (Sc35)**	CGAAGCGAGAGTCCAAGTCT	GCCACCTGAGGCAGATTAAA
***Srsf1* (Sf2/Asf)**	CCTTCGTTGAGTTCGAGGAC	TTTAAGTCCTGCCAGCTTCC
Tnfa	CGAGTGACAAGCCTGTAGCC	GTGGGTGAGGAGCACGTAGT
Il1β	TGAGCACCTTCTTTTCCTTCA	CGTTGCTTGGTTCTCCTTGT
Gapdh	AACTTTGGCATTGTGGAAGG	ACACATTGGGGGTAGGAACA

4.6.3 Phospholipid, sterol and hyperoxide (FOX1) assays

Lipid profiling of the samples in study IV was performed by our collaborators at the University of Szeged, Hungary, and the FOX1 assay were done by prof. Rashid Giniatullin's group at A.I.V. Institute of University of Eastern Finland, and are described in detail in the original publication (Koivisto et al., 2014). Briefly, the levels of phosphatidylcholine (PC) and phosphatidylethanolamine (PE) and their derivatives were determined from the cortices of saline-perfused mice using reversed-phase contrast liquid chromatography (Kinetex C18) and mass spectrometry (Waters Q-TOF Premier). Sterols and oxysterols were measured from both the liver and temporal cortex of non-perfused mice with gas-liquid chromatographic and mass spectrometric (GC-MS; HP5890 Series II plus gas chromatograph and HP5971 mass selective detector) analysis. The concentrations of lipid hydroperoxides in the cerebellum and hippocampus were determined using ferrous ion oxidation in an xylenol orange assay and by reading sample absorbance at 560 nm (FOX1) (Banerjee et al., 2003, Jiang et al., 1992).

4.6.4 Protein extraction and Western blot

Protein extraction from the frozen human brain tissue samples used in study II was conducted previously and described in detail in Natunen et al., 2013b (Natunen et al., 2013b). Briefly, total proteins were extracted from the organic phase (phenol-ethanol supernatant) obtained after extraction of DNA (Natunen et al., 2013a, Natunen et al., 2013b). The organic phase was incubated with three volumes of acetone at room temperature for 10 min. The protein fraction was then separated by centrifugation at 10000 *x g* for 10 min. The pellet was re-suspended for 20 min before being washed two times with wash buffer (0.3 M guanidine hydrochloride in 1:1 mixture of 95% ethanol and 2.5% glycerol) at room temperature. The final wash was performed with ethanol, supplemented with 2.5% glycerol. The samples were centrifuged at 8000 *x g* for 5 min and the protein pellets were air-dried for 10 min at room temperature. Pellets were dissolved in UREA-CHAPS [9.5 M urea and 2% 3-[(3-cholamidopropyl)-dimethylammonio]propanesulfonate, pH9.1] by shaking for 15 min.

Total proteins from mouse temporal cortex homogenates (in PBS supplemented with EDTA-free protease and HALT™ phosphatase inhibitor cocktails) or cultured cells were extracted by using tissue protein extraction buffer (TPER; Pierce) supplemented with EDTA-

free protease and HALT™ phosphatase inhibitor cocktails, as follows. One volume of TPER was added to mouse tissue homogenates and incubated on ice for 30 min to allow cell lysis. Cells in culture were washed twice with PBS on ice and scraped into TPER. After a 30-min incubation, mouse brain tissue samples and cells were centrifuged at 10000 *x g*, for 10 min, at +4˚C to separate the total protein fraction.

Total protein concentrations were measured with the BCA protein assay kit (Pierce), according to manufacturer's instructions. Ten to fifty μg of denatured protein lysates in NuPAGE LDS sample buffer (Invitrogen) containing 20% β-mercapto-ethanol, were subjected to 4-12% Bis-Tris polyacrylamide gel electrophoresis (Invitrogen) and transferred onto polyvinylidene difluoride membranes (Amersham Hybond-P, GE Healthcare). Non-specific antibody staining was prevented with preincubation of the membranes with solutions containing 5% of either non-fat milk powder or BSA. Membranes were incubated with primary antibodies overnight at +4˚C (**Table 7**). Next day, membranes were washed and incubated with anti-mouse, anti-rabbit or goat anti-rabbit horseradish peroxidase (HRP)-conjugated secondary antibody for 1 h at room temperature. Protein bands were detected using enhanced chemiluminescence substrates (ECL, Amersham, GE Healthcare) and an ImageQuant RT ECL camera (GE Healthcare). Protein band intensities were quantified by using Quantity One (BioRad) software and normalized to those of GAPDH or β-actin in the same samples.

4.6.5 Immunoprecipitation

Tissue homogenates of *UBQLN1* TV1- and GFP-injected mouse hippocampi were adjusted to 20 μg with immunoprecipitation (IP) buffer (50 mM Tris pH 8.0, 150 mM NaCl, 2 mM EDTA, 0.5% Igepal) containing Complete Mini EDTA-free protease inhibitor (Roche) in a final volume of 500 μl. Protein samples were incubated with Protein A/G Agarose beads (Pierce) without antibody for 1 h on a rotating wheel to remove unspecifically binding proteins. Samples were centrifuged at 8000 *x g* for 1 min at 4˚C. Pre-cleared supernatants were immunoprecipitated with 2.5 μl of anti-APP (A8717) antibody for 1 h at +4°C on a rotating wheel. Fresh A/G Agarose beads were added to the samples and rotated overnight at +4˚C, to pull down the protein-antibody complexes. The beads were harvested by centrifugation for 1 min at 8000 *x g* at 4˚C, and washed four times with IP buffer. Precipitated proteins were detached by heating at 55˚C for 10 min in NuPAGE LDS sample buffer containing 5% β-mercaptoethanol. The samples were then centrifuged at 13000 *x g* for 15 min and the supernatants were analysed with Western blot. Samples without antibody (beads only) were used as a negative control and a sample with a rabbit polyclonal antibody known not to interact with APP was used as a control for specificity of the assay.

4.6.6 Aβ measurement

Mouse brain homogenates from the hippocampus (study II) or temporal cortex (study IV), were used for Aβ measurements. In study IV, Aβ levels were determined by Prof. Heikki Tanila's group at the A.I.V. Institute at the University of Eastern Finland with a protocol described in detail in the original publication (Koivisto et al., 2014). In study II, omogenates in PBS were centrifuged at 100000 *x g* for 2 h at +4˚C (Optima L-90K Ultracentrifuge, Beckman) to obtain soluble and insoluble fractions. Insoluble pellets were solubilized in 5M guanidine-HCl, including 50 mM Tris-HCl, pH 8.0 by vortexing for 3 h at room temperature

and centrifuged at 10000 *x g* for 10 s. Aβ x-40 and Aβ x-42 levels were measured from soluble and insoluble fractions by using Human/Rat β Amyloid x-40 and Human/Rat β Amyloid x-42 ELISA kits (Wako or Biosource International). Reactions were terminated after a 30-min incubation at room temperature and absorbance was measured at wavelength 450 nm using an ELISA microplate reader (BioRad). Aβ levels were normalized to total protein concentrations and APP levels in the same samples.

4.6.7 β- and γ-secretase activity assays

In study IV, β- and γ-secretase activities were determined from the left cortex homogenates by the collaborators at the University of Saarland, Germany, with a protocol described in the original publication (Koivisto et al., 2014). In study II, 1.5 μg of hippocampal homogenates in PBS were incubated with 150 mM β-secretase-specific substrate conjugated to fluorescent reporter molecules EDANS and DABCYL, for 1 h at +37°C. The β-secretase activity was inhibited from randomly selected samples with 150 mM GL186 (H-VENstatineVAEF_NH_2, Calbiochem) to control the specificity of the assay. Emitted light was detected at the wavelength, 510 nm after excitation at 355 nm with a fluorescence microplate reader. Readings obtained from substrate alone (no secretases) were subtracted from the sample values.

4.6.8 Extracellular tau measurement

Extracellular tau levels were measured from mouse primary neuron – microglial cell co-culture medium by using the mouse tau ELISA kit (Invitrogen) performed according to manufacturer's instructions. The reactions were terminated after 30 min incubation and absorbance was measured at the 450 nm wavelength with ELISA microplate reader (BioRad). Extracellular tau levels were normalized to the total protein concentration and neuronal viability in each sample.

4.6.9 TNFα and IL1β measurements

Mouse temporal cortical tissue homogenates without inhibitors (Study III) were centrifuged at 100000 *x g* for 45 min at +4°C (Optima L-90K Ultracentrifuge, Beckman) to separate soluble and insoluble proteins. TNFα and IL1β levels were measured from soluble supernatant fraction by using mouse TNFα (Ready-Set-Go!, eBioscience) and mouse IL1β (Abcam) ELISA kits. Reactions were terminated after a 30 min incubation and absorbance was measured at wavelength 450 nm with an ELISA microplate reader. TNFα levels were measured from the medium (150 μl) of mouse primary neuron and BV2 microglial cell co-cultures, as described above (study II). TNFα and IL1β levels were normalized to the total protein concentrations from the same samples.

4.6.10 Neuronal viability

Mouse primary cortical neuron and BV2 microglial cell co-cultures were fixed in 4% paraformaldehyde (PFA) solution for 20 min at room temperature, rinsed twice with PBS and permeabilized with a solution containing methanol as well as 0.3% H_2O_2 for 10 min. Non-specific antibody staining was prevented with immersing the samples in blocking buffer (PBS, 1% BSA, 10% horse serum) for 20 min at room temperature. Cells were then incubated with neuron-specific MAP2 primary antibody (1:2000 in blocking buffer, **Table 8**) overnight

at +4˚C (Gresa-Arribas et al., 2012). The next day, cells were washed three times with PBS and stained with biotinylated horse anti-mouse secondary antibody (1:500 in blocking buffer; Vector Labs) for 1 h at room temperature. This was followed by three washes with PBS and incubation with ExtrAvidin-HRP (1:500 in blocking buffer; Sigma) for 1 h at room temperature. To develop the color, cells were incubated with ABTS peroxidase substrate (Vector Labs) in the dark, for 30 min. Wavelength 405 nM absorbances were measured on a fresh 96-well plate with an ELISA microplate reader (BioRad).

4.7 STATISTICAL ANALYSES

Microarray analysis of the neuropathological sample cohort was carried out with R statistical software (version 3.0.1) and Bioconductor (version 2.13), and is described, at length, in Martiskainen et al., 2015 (Martiskainen et al., 2015). The expression analysis of *UBQLN1* was performed using a Kruskal-Wallis test (for data which is not normally distributed). Statistical analysis of functional studies were carried out using specific tests contained within either GraphPad Prism or SPSS software (version 21.0). Analysis of variance (ANOVA), followed by the appropriate post-hoc test, was used in experiments with more than one variable. In experiments with only one variable, an independent sample's t-test (equal variance assumed) or Mann-Whitney U test (equal variance not assumed) was used to test the statistical significance between sample groups. Pearson's two-tailed correlation analysis or Spearman's correlation coefficient's were used to analyze bivariate correlations between parameters of interest. All results are reported as means ± standard deviation (SD) or standard error of mean (SEM). The level of statistical significance was defined as $p<0.05$.

5 Results

5.1 INFLUENCE OF UBIQUILIN-1 ON DIFFERENT ASPECTS OF ALZHEIMER'S DISEASE PATHOGENESIS: STUDIES I AND II

Cell stress and impairment of certain basic cellular functions, such as protein trafficking and quality control systems, have been implicated to play a role in early AD pathogenesis (Ross and Poirier, 2004) (Wang et al., 2014). Ubiquilin-1 is a protein which has been shown to regulate the trafficking, accumulation and clearance of several AD-associated proteins as well as alleviate cellular stress conditions (Haapasalo et al., 2010) (El Ayadi et al., 2012b) (Hiltunen et al., 2006, Ko et al., 2002, Liu et al., 2014, Lu et al., 2009). Study I aimed to review the function of ubiquilin-1 and discuss its potential as a therapeutic target in AD. In study II, the goal was to investigate how ubiquilin-1 affects different aspects of AD-related pathological pathways in human brain and in experimental cell and mouse models of AD.

5.1.1. Ubiquilin-1 expression is decreased, with respect to the severity of neurofibrillary pathology, in the human brain.

Data regarding ubiquilin-1 in human brain and in mammalian animal models is scarce. In study II, the expression of ubiquilin-1 was first investigated in *post mortem* human brain samples, in relation to AD-related neurofibrillary tangle pathology (Braak et al., 2006). For expression analysis, sixty previously characterized (Martiskainen et al., 2015, Natunen et al., 2013a, Natunen et al., 2013b) human temporal cortex samples were divided into three groups: Braak stage 0-II (n=28), Braak stage II-IV (n=13), and Braak stage V-VI (n=19). Expression of individual *UBQLN1* exons (exons 4, 6, 9, and 11) was assessed, after being normalized to the global expression of the *ACTBL2* gene. The analysis revealed a global decrease in *UBQLN1* mRNA expression in relation to advancing neurofibrillary pathology. Overall, the highest *UBQLN1* expression was observed in Braak stage 0. The expression of exons 4, 6, and 9 were significantly ($p<0.05$) lower in Braak stage III-IV, as compared to Braak stage 0-II, whereas expression of exon 11 was significantly ($p<0.05$) lower in Braak stage V-VI than in Braak stage 0-II. Furthermore, a similar, although not statistically significant, decrease in ubiquilin-1 protein levels was observed in Braak stages III-IV (n=7) and V-VI (n=7), as compared to Braak stage 0-II (n=10). These data are in line with a previous study showing that ubiquilin-1 levels are decreased in AD brain as compared to age-matched controls (Stieren et al., 2011). Since previous reports suggest that ubiquilin-1 may modulate APP processing *in vitro* and *in vivo* (Hiltunen et al., 2006, Stieren et al., 2011, Zhang et al., 2007) (El Ayadi et al., 2012b), the association between ubiquilin-1 levels and the levels of key proteins in APP processing was next assessed. As a result, a trend of positive correlation between the protein levels of ubiquilin-1 and BACE1 in human brain was found. However, there was no association between ubiquilin-1 levels and β-secretase activity.

5.1.2 Ubiquilin-1 overexpression decreases neuronal viability and increases BACE1 protein levels in embryonic mouse cortical neuron and BV2 microglial cell co-cultures

Previous studies suggest that ubiquilin-1 may alleviate oxidative and ER stress *in vivo* and in cultured cells (Kim et al., 2008, Lim et al., 2009, Liu et al., 2014, Lu et al., 2009). However, the

role of ubiquilin-1 during AD-associated neuroinflammation has not been investigated (Heneka et al., 2015). To delineate the effect of ubiquilin-1 overexpression on different aspects of AD-pathology under neuroinflammation, primary neuron cultures from embryonic stage 18 mouse cortices were prepared. Lentivirus-mediated transductions with the full-length human *UBQLN1* TV1 cDNA or control lentivirus, which lacks the coding insert, were performed on DIV4. A day later, BV2 microglial cells were added to the neuronal cultures and co-cultures were treated with LPS (200ng/ml) + IFN-γ (5ng/ml) for 48 h to induce neuroinflammation (**Figure 10A**). First, the amount of secreted TNFα was analyzed from the medium, to evaluate the extent of neuroinflammation. TNFα levels were significantly increased after LPS + IFN-γ-treatment as compared to vehicle treated samples ($p<0.0001$, n=15). In spite of an evident inflammatory response, neuronal viability was only slightly compromised after LPS + IFN-γ treatment. Western blot analysis revealed that protein levels of human ubiquilin-1 were approximately ~3.5 (in vehicle treated samples, $p<0.01$, n=3-4) to 5 (in LPS + IFN-γ treated samples, $p<0.01$, n=3-4) fold increased in cell transduced with the TV1 of human *UBQLN1* gene as compared cells transduced with control virus. Interestingly, overexpressed human ubiquilin-1 levels were also significantly higher in LPS + IFN-γ-treated samples than in vehicle-treated samples, suggesting that *UBQLN1* expression might be upregulated during neuroinflammation ($p<0.05$). However, a similar increase in ubiquilin-1 protein levels was not observed in endogenously expressed mouse *Ubqln1* gene. A slight, approximately 30%, increase in TNFα levels in the medium of LPS + IFN-γ treated samples was observed upon ubiquilin-1 overexpression, although the difference to control virus was not statistically significant. Surprisingly, ubiquilin-1 overexpression significantly decreased neuronal viability in both vehicle-treated and LPS + IFN-γ-treated cultures as compared to control virus (LPS + IFN-γ-treated, $p<0.05$; vehicle-treated, $p<0.0001$, n>10). To address the possible mechanism, by which ubiquilin-1 might affect neuronal survival, the activation of Akt signaling pathway, which is known to affect neuronal survival, was next assessed in co-cultures. Akt phosphorylation (p-Ser473) was significantly increased after LPS + IFN-γ-treatment regardless of the transduction ($p<0.05$, n=3-4), suggesting that LPS + IFN-γ-induced neuroinflammation increases Akt signaling. However, there were no significant differences upon ubiquilin-1 overexpression in the phosphorylation status of Akt, suggesting that compromised neuronal viability is not mediated by altered activity of the Akt signaling pathway. Together, the data suggest that ubiquilin-1 does not have a major influence on the level of neuroinflammation, but ubiquilin-1 overexpression itself appears to be detrimental for mouse cortical neurons. The augmented neuronal death upon ubiquilin-1 overexpression is not likely to be mediated via changes in the Akt signaling pathway in this case.

Driven by the possible interrelationship between ubiquilin-1 and BACE1 in the aged human brain, the effect of ubiquilin-1 overexpression on the levels of endogenously expressed BACE1 was next investigated. Interestingly, ubiquilin-1 overexpression induced a significant increase in BACE1 levels as compared to control ($p<0.05$, n>10). Previous studies have shown that BACE1 expression is promoted under inflammation after treatment with proinflammatory compounds, such as LPS and IFN- (Cho et al., 2007, Wang et al., 2015). In line with this, a trend of increased BACE1 protein levels after LPS + IFN-γ-treatment was detected in study II, although it was not statistically significant.

Ubiquilin-1 has previously been shown to co-localize in NFTs in AD brain (Mah et al., 2000), but the interrelationship tau and ubiquilin-1 has not been further investigated. Therefore the effect of ubiquilin-1 overexpression under neuroinflammation on tau phosphorylation, total tau protein levels, and tau secretion was also assessed. Ubiquilin-1 overexpression, *per se,* did not exert major effects on tau phosphorylation, protein levels or secretion in this *in vitro* model. However, total protein levels of the 0N3R-tau isoform were significantly increased by the LPS + IFN-γ treatment in ubiquilin-1 overexpressing cells, but not in control cells, suggesting that ubiquilin-1 may modulate tau levels under inflammatory conditions ($p<0.01$, $n>9$). Tau phosphorylation has previously been shown to be increased after LPS treatment *in vivo* (Kitazawa et al., 2005, Nikkel et al., 2012), but, here, no significant alterations in tau phosphorylation were detected at AD-related epitopes (AT8) in co-cultures by LPS + IFN-γ-treatment or ubiquilin-1 overexpression. Despite this, since the results implied that there might exist a relationship between ubiquilin-1 and tau, the effect of ubiquilin-1 overexpression on the predominant adult 0N4R-tau isoform was evaluated in the HEK293-AP-APP cell line. In these cells, myc-TV1-mRFP overexpression induced a significant increase in the phosphorylation (AT8, $p<0.01$, $n=8$) and total protein levels ($p<0.05$, $n=8$) of co-overexpressed 0N4R-tau as compared to cells co-overexpressing control plasmid (mRFP) and 0N4R-tau. Therefore, further studies are needed to assess the detailed mechanism of the potential interaction between tau and ubiquilin-1.

5.1.3 Ubiquilin-1 overexpression moderately increases BACE1 levels and activity in adult APdE9 mice hippocampi

Next, human *UBQLN1* TV1-containing lentiviruses were bilaterally injected into hippocampi of five-month old male mice (n=7) to study the effect of ubiquilin-1 overexpression *in vivo* in the APdE9 mouse model of AD. Sham-operated mice (n=8) and mice injected with GFP-containing lentiviruses (n=8) were used as controls. Western blot analysis and IHC assessment showed cleared ubiquilin-1 protein expression in the granular cells of the dentate gyrus of hippocampus of TV1-injected mice eight weeks (a pilot study) and 4.5 months after the injection. In contrast, ubiquilin-1 staining was detected neither in GFP-injected mice nor in the cortex of ubiquilin-1 TV1-lentiviral injected mice, suggesting that ubiquilin-1 overexpression was successfully induced and that its expression was restricted to hippocampal area.

The effect of ubiquilin-1 overexpression on BACE1 levels and activity in APdE9 mice brain was next addressed. Here, BACE1 protein levels and β-secretase activity were mildly, but consistently increased in the hippocampi of TV1-injected mice as compared to GFP-injected mice. This did not, however, reach statistical significance. BACE1 and ubiquilin-1 were partially co-localized in the cells of dentate gyrus, although their subcellular localization was different as BACE1 was mostly localized in puncate structures, which did not include ubiquilin-1. Strong BACE1 staining was also observed around Aβ plaques and this was not changed due to ubiquilin-1 overexpression. The β-secretase activity was significantly correlated with soluble $A\beta_{40}$ and $A\beta_{42}$ levels in the hippocampi of APdE9 mice ($p<0.001$). Ubiquilin-1 has previously been shown to alter APP maturation, trafficking, processing, and degradation in cultured cells (El Ayadi et al., 2012a, Hiltunen et al., 2006, Stieren et al., 2011, Viswanathan et al., 2011, Viswanathan et al., 2013, Zhang et al., 2007). In

study II, no significant changes were observed in the APP maturation, levels of APP metabolites (CTFs and Aβ), plaque load, or APP ubiquitination status in the hippocampi of TV1-injected mice as compared to GFP-injected mice. However, plaque load was modestly decreased, while soluble and insoluble $A\beta_{40}$, as well as soluble $A\beta_{42}$ levels, were increased in the hippocampi of TV1-containing lentivirus injected mice as detected with IHC and ELISA, respectively. It is plausible, though, that this variability arises from a dynamic nature of Aβ aggregates, in other words, by constant aggregation and dissolution of Aβ molecules from the plaques, as described previously (Maggio et al., 1992). Finally, no significant alterations in tau phosphorylation (AT8) or total tau protein levels were detected in TV1-injected mice as compared to GFP-injected mice. These data altogether suggest that ubiquilin-1 does not exert major effects on tau levels or APP processing or degradation *in vivo* in the APdE9 mouse hippocampus. However, it may slightly affect BACE1 levels, β-secretase activity as well as Aβ levels and plaque load in these mice. Moreover, ubiquilin-1 overexpression does not appear to influence neuroinflammation *in vivo* in the mouse brain, as the levels of glial fibrillary acidic protein (GFAP) and the CD45 immunopositive area remained unaltered between TV1- and GFP-injected mice.

5.1.4 Ubiquilin-1 overexpression increases BACE1 stability by diverting it from the lysosomal pathway in cultured neuronal cells

To mechanistically characterize the observed interrelationship between ubiquilin-1 and BACE1, H4 cells stably overexpressing human ubiquilin-1 TV1 (H4-TV1) and control cells stably expressing empty plasmid (H4-pcDNA) were transiently transfected with BACE1 plasmids under different promoters (5'UTR-BACE1, 5'3'UTR-BACE1 or BACE1-myc). Transfection of the BACE1-myc plasmid containing highly potent CMV promoter, but without endogenous 5'UTR or 3'UTR BACE1 regulatory regions, yielded the highest BACE1 overexpression in this series of experiments. Expression of BACE1 plasmids containing the endogenous promoter and 5'3'UTR or 5'UTR regulatory regions resulted in a more moderate BACE1 overexpression. Cells transfected with the control plasmid (pcDNA) did not show BACE1 protein in Western blots, indicating that H4 cells do not endogenously express detectable amounts of BACE1. Ubiquilin-1 overexpression significantly increased BACE1 expression levels in all cells transfected with the three different BACE1 constructs: BACE1-myc, 5'UTR-BACE1 and 5'3'UTR-BACE1 levels were ~1.7 fold ($p<0.05$), ~3.3 ($p<0.01$) fold and ~4.0 fold ($p<0.01$) increased, respectively, in H4-TV1 cells as compared to H4-pcDNA cells ($n=7$). This finding was further corroborated by the fact that ubiquilin-1-mediated increase in BACE1 levels was reversed by siRNA-mediated downregulation of overexpressed ubiquilin-1 in H4-TV1 cells. Here approximately a ~20% reduction in *UBQLN1* TV1 levels ($p<0.05$) by RNAi resulted in approximately ~15% decrease in 5'3'UTR-BACE1 levels ($p<0.05$, $n=4$). Furthermore, correlation analysis revealed a statistically significant positive correlation between protein levels of ubiquilin-1 and BACE1, both before and after ubiquilin-1 downregulation, suggesting a strong association between these proteins ($p<0.01$, $n=6$-8). A similar significant increase in BACE1 levels was also observed in two additional cell lines transiently transfected with ubiquilin-1 TV1 and 5'3'UTR-BACE1 ($p<0.001$ and $p<0.01$ in H4-APP751 and SH-SY5Y-APP751, respectively). This confirmed that ubiquilin-1 induced an increase in BACE1 levels that was not restricted to particular cell types. Furthermore,

ubiquilin-1 overexpression did not exert a similar increase in the levels of co-overexpressed EGFP-F, or endogenously expressed ERK1/2 protein, suggesting a specific interrelationship between ubiquilin-1 and BACE1. These data collectively indicate that both transient and stable co-overexpression of ubiquilin-1 with BACE1 significantly and specifically increases BACE1 protein levels in different neuronal cell lines.

The next aim was to investigate potential molecular mechanism(s) that may account for the observed interrelationship between ubiquilin-1 and BACE1. First of all, a cycloheximide time-course assay was performed to study whether ubiquilin-1 affects the half-life of BACE1. This assay revealed significantly ($p<0.001$, $n=3$) prolonged half-life of 5'3'UTR-BACE1 in H4-TV1 ($t_{1/2}$ ~7.5h) cells as compared to H4-pcDNA cells ($t_{1/2}$ ~5h). These findings suggest that overexpression of ubiquilin-1 increases BACE1 levels by slowing down the degradation of BACE1. To investigate further how the decelerated degradation could be explained, SH-SY5Y-APP751 cells were co-transfected with 5'3'UTR-BACE1 and myc-TV1 or 5'3'UTR-BACE1 and control plasmid (pcDNA) and investigated by microscopy. Myc-TV1 localization was mainly cytoplasmic and it partially resided inside large cytoplasmic structures that may contain ubiquitinated proteins, as shown previously in these cells (Viswanathan et al., 2011). BACE1 did not co-localize with myc-TV1 in these structures. Similar to the *in vivo* data, BACE1 was localized inside small vesicles in the soma and processes of the cells as indicated by punctate staining. BACE1 has previously been shown to localize in endosomes and lysosomes (Cole and Vassar, 2007). In accordance with this information, BACE1 prominently co-localized with Rab7, a marker for LEL, in these cells. BACE1 displayed co-localization as well with transferrin receptor (TfR), a marker for early and recycling endosome and EEA1, a marker for early endosomes. Myc-TV1 overexpression significantly decreased BACE1 co-localization with Rab7 when compared to control cells (pcDNA) ($p<0.05$, $n>45$). A concominant increase in BACE1 co-localization with TfR was observed in cells overexpressing myc-TV1 as compared to control cells, but this increase did not reach statistical significance ($n>13$). Similarly, BACE1 co-localization with EEA1 in cells overexpressing myc-TV1 showed an increasing, but statistically non-significant, trend. No evidence for altered protein levels of several different factors known to affect BACE1 subcellular trafficking or sorting, such as GGA1, GGA3, ARF6, seladin-1, or BACE1 Lys-48 or Lys-63-linked ubiquitination, were detected. Despite this, the data suggest that overexpression of ubiquilin-1 leads to decreased localization of BACE1 in the lysosomal compartment, where BACE1 degradation normally takes place.

5.2 MOLECULAR AND BEHAVIORAL EFFECTS OF DIETARY MANIPULATION IN MICE: STUDIES III AND IV

Several lines of evidence suggest that dietary fatty acid composition may play a role in AD risk and several aspects of AD pathogenesis (Morris and Tangney, 2013). It has been suggested that 'typical Western-type' HFD, containing considerable amount of SFAs, may increase the risk and/or progression of the AD pathology (Ho et al., 2004, Julien et al., 2010, Kohjima et al., 2010, Laitinen et al., 2006, Leboucher et al., 2013, Morris et al., 2003a, Oksman et al., 2006). On the other hand, a growing body of evidence implies that diets rich in PUFAs and *n-3* sources, such as fish oil, may alleviate amyloid pathology and reduce the risk of dementia (Laitinen et al., 2006, Morris et al., 2003b, Morris et al., 2015, Oksman et al., 2006).

Moreover, it has been proposed that additional dietary supplements, including plant sterols and nutrients consisting of precursors and cofactors for neuronal membrane synthesis may enhance the beneficial effects of fish oil-based diet. Studies III and IV, aimed to investigate the effects of HFD and special fish oil-based diets on AD-related molecular and behavioral changes in mice.

5.2.1 A high-fat diet increases tau expression and exon 10 inclusion in mouse brain regardless of peripheral metabolic status: Study III

AD and T2DM are risk factors for each other, which both are influenced by environmental factors, such as diet (Carlsson, 2010, Morris and Tangney, 2014, Sindi et al., 2015). Our group has previously investigated this co-morbidity in mice concomitantly engineered to model both of these disorders by genetic and dietary manipulation (Hiltunen et al., 2012). For that purpose, the APdE9 mice (A+Iw) were crossbred with the T2DM mice, overexpressing *IGF2* in the pancreas (AwI+), to create triple transgenic APdE9xIGF2 mice (A+I+). In addition, mice in each genotype group (AwIw, A+Iw, AwI+, A+I+) were fed with HFD for four months. AwIw, A+Iw, AwI+, and A+I+ mice fed with standard laboratory mouse food (STD) were used as controls. In the previous study, tau protein levels and phosphorylation (at the AT8 epitopes Ser199, Ser202/Thr205) were demonstrated to be increased in the brain of female mice after HFD, while Aβ load was unaffected by the diet or *IGF2* genotype (Hiltunen et al., 2012). In line with these previous data, a robust increase in the protein levels of 4R-tau isoform was detected in the temporal cortex of female mice on HFD as compared to those on STD in study III. Accordingly, a similar, approximately ~4.5 fold significant increase in 4R-tau mRNA levels was observed in the temporal cortex of female mice on HFD, as compared to those on STD ($p<0.0001$). No such diet-effect was detected in 4R-tau protein or mRNA levels in the temporal cortex of male mice, indicating that diet-induced changes in tau expression are dependent on gender-related factors, such as female hormones. In female mice, both protein and mRNA levels of 4R-tau were unaltered upon the presence of AwIw, A+Iw, AwI+, or A+I+ genotype. This suggests that a high-fat diet increases 4R-tau expression regardless of AD or T2DM genetic background.

3R-tau protein levels in the temporal cortex of adult mice were too low for detection with Western blot. Despite 3R-tau mRNA levels being, similarly, extremely low, a significant increase in the 3R-tau mRNA expression was detected in the temporal cortices of HFD-fed female mice as compared to STD-fed female mice ($p<0.0001$). However, as the HFD-induced increase in the 3R-tau mRNA expression (~30%) was much less pronounced than that seen with the 4R-tau (~400%), a significant increase in 4R-tau/3R-tau mRNA ratio was observed in the temporal cortex of female mice fed a HFD ($p<0.0001$). This suggests that a HFD not only increases tau expression, but also may affect alternative splicing of exon 10. In contrast to 4R-tau, 3R-tau mRNA expression was additionally affected by the genotype to diet interaction in female mice. More precisely, 3R-tau mRNA expression was significantly higher in triple transgenic A+I+ mice on STD as compared to A+Iw and AwI+ mice on STD, but this increase was not further potentiated by HFD. The finding implies that expression of 4R-tau and 3R-tau isoforms might be regulated via different mechanisms in the mouse brain. Notably, the HFD-induced increase in tau expression was found to be associated with a cognitive phenotype, as demonstrated in the previous study (Hiltunen et al., 2012). In other

words, the mRNA levels of 4R-tau and 3R-tau significantly correlated with impaired spatial memory (search bias) in both the swim navigation task (r=-0.38 and r=-40, respectively; $p<0.05$ for both) and the reduced vertical activity (rearing) measurement (r=-40 and r=-36, respectively; $p<0.05$ for both).

Additionaly, 4R-tau and 3R-tau mRNA levels, as well as the 4R-tau/3R-tau mRNA ratio correlated with diet-induced weight gain, peripheral metabolic changes, the levels of neuroinflammatory markers and the activity Akt/glycogen synthase kinase 3β (GSK3β) signaling cascade. HFD exposure induced a significant weight gain in all genotypes except in A+Iw ($p<0.0001$). The correlation analysis revealed a significant positive association between weight gain and the mRNA levels of 4R-tau (r=0.71; $p<0.01$), 3R-tau (r=0.46; $p<0.01$) and 4R-tau/3R-tau ratio (r=0.62; $p<0.0001$). However, as the A+Iw mice did not gain weight while put on the HFD, the weight gain is not probably the only mechanism explaining the genotype-independent increase in tau expression after HFD. In the previous study, both the *IGF2* genotype (I+) and the HFD induced peripheral hyperglycemia, whereas sustained hyperinsulinaemia and insulin resistance were only observed in triple transgenic (A+I+) mice, regardless of the diet (Hiltunen et al., 2012). Accordingly, no correlation was found between the mRNA levels of 4R-tau, 3R-tau or 4R-tau/3R-tau ratio and blood β-glucose or plasma insulin levels (as determined by GTT and ITT, respectively) in female mice. HFD significantly affected neither the levels of astrocyte marker protein GFAP nor CD45 immunopositive area, which was determined in the previous study (Hiltunen et al., 2012). In line with this, expression-wise tau changes were not found to correlate with the levels GFAP. In contrast, HFD significantly decreased the protein levels of TNFα, and slightly, but insignificantly decreased the protein levels of IL1β, while the mRNA levels of both inflammatory cytokines remained unaltered. TNFα and IL1β protein levels were negatively correlated with mRNA levels of 4R-tau (r=-0.36, $p<0.05$ and r=-0.42, $p<0.05$, respectively) and 4R-tau/3R-tau mRNA ratio (r=-0.40, $p<0.05$ and r=-0.46, $p<0.01$, respectively). Together, these observations imply that HFD-induced tau expression is not associated with accelerated neuroinflammation in the temporal cortex of female mice.

Next, the effect of HFD on the activity of the Akt/GSK3β signaling pathway was determined. However, the activities of these two central kinases involved in insulin signaling and tau phosphorylation were not influenced by the diet or associated with diet-induced tau changes. Finally, expressional changes of established tau exon 10 splicing enhancers, SF2/ASF, C35, Tra2β, and Sp30c, were elucidated to address the mechanism behind increased 4R-tau to 3R-tau mRNA ratio (Liu and Gong, 2008, Qian and Liu, 2014). However, there were no significant diet-related changes in the mRNA levels of any of these splicing agents examined. In summary, these results suggest that a high-fat diet increases tau expression and exon 10 inclusion in female mice, regardless of the animal's genotype, peripheral or central insulin signaling, or neuroinflammation status.

5.2.2 Special fish oil based diets alleviate cognitive deficits in the APdE9 mice independent of β amyloid deposition: Study IV

To investigate potential beneficial effects of dietary fish oil and specific additional nutrients in a mouse model of AD, APdE9 female mice were fed with diets containing fish oil (FO), fish oil and plant sterols (FOPS), or fish oil and precursors and cofactors for synaptic

membrane synthesis (Fortasyn)(**Table 6**), for 9 months. APdE9 mice and their wild-type littermates fed with normal laboratory mouse chow were used as controls in the study. In line with the previous reports, 14-month-old wild-type mice performed significantly better in the swim navigation task than age-matched APdE9 mice on the control diet ($p<0.05$). None of the experimental diets (FO, FOPS, Fortasyn) improved spatial learning of the APdE9 mice, as was indicated by the similar escape latencies between diet groups during the five task acquisition days. However, when the two last aquisition days were analyzed separately (the learning curve had reached the plateau), the mice on Fortasyn diet performed significantly ($p<0.05$) better than FO or FOPS fed mice. This suggests that fish oil, in combination with specific nutrients targeted for retaining synaptic function, may be more efficient in improving spatial memory than fish oil alone.

Olfactory dysfunction is one of the earliest symptoms of AD (Masurkar and Devanand, 2014) and has been reported to occur in transgenic mouse models of AD as well (Cassano et al., 2011, Wesson et al., 2011). Therefore, the ability to distinguish species-specific odors was tested next. Odor recognition ability of the mice was determined as the relative time, which each mouse spent exploring two wooden balls, one odorized with the scent of an unfamiliar mouse (novel odor) and the other one with the mouse's own odor (familiar odor). Wild-type mice were significantly more interested in exploring the wooden balls than APdE9 mice on control diet ($p<0.05$). Wild-type mice were also clearly more interested in the novel odor than of the familiar one as they spent approximately 70% of the total time exploring the novel odor. In contrast, APdE9 mice on control diet did not show an odor preference at all and, thus, their behavior was significantly different from that seen in the wild-type litter-mate mice ($p<0.05$). Importantly, each of the experimental diets fully restored the odor recognition ability in APdE9 mice. The mice on FO, FOPS and Fortasyn diets showed a strong preference towards the novel odor and each group spent more than 70% of total time exploring the ball with the scent of unfamiliar mouse (FO $p<0.01$, FOPS $p<0.05$, Fortasyn $p<0.01$, as compared to control group). This indicates that AD-associated olfactory deficit in mice may be alleviated by long-term fish oil consumption.

Fear and anxiety-related hyperactivity and decreased explorative behavior are characteristics of the APdE9 model genotype when these animals are placed in a novel environment (Jansen et al., 2013) (Kemppainen et al., 2012). Accordingly, significantly ($p<0.01$) increased activity (ambulatory distance) among APdE9 mice on control diet as compared to wild-type mice was observed in study IV. However, none of the experimental diets was found to reduce the hyperactive behavior of APdE9 mice. Habituation to the novel environment was determined by comparing the time that the mouse spent exploring the test cage (rearing time) on day 3 versus on the first trial day. APdE9 mice on control diet habituated poorly and were the least similar with the wild-type mice in their behavior. FOPS-fed mice, on the contrary, showed good habituation, as they spent significantly less time rearing on the third trial day than on the first trial day ($p<0.05$). However, FOPS-fed mice were also significantly heavier than APdE9 mice on control diet ($p<0.0001$). It is therefore possible that increased body weight affected the rearing activity of FOPS-fed mice.

The β-secretase activity has been shown to increase in the AD brain (Vassar et al., 2014). In line with this, β-secretase activity proved to be significantly increased in the brain of APdE9 mice on control diet as compared to wild-type mice on control diet ($p<0.01$). However,

no similar genotype effect was observed on the activity of γ-secretase. Previous *in vitro* and *in vivo* studies have suggested that DHA-derived n3 PUFAs may reduce Aβ load by inhibiting the activities of both β- and γ-secretase (Grimm et al., 2011). Agreeing with this, a significant effect of the dietary manipulation was observed on both β-secretase ($p<0.001$) and γ-secretase ($p<0.01$) activity. However, the previously reported DHA activity, here present in all experimental diets, was also strongly modified by other dietary components. More precisely, only the FO diet significantly reduced the activity of β-secretase in the cortex as compared to APdE9 mice on control diet ($p<0.001$). A similar trend was also observed in the cortex of Fortasyn-fed mice, although this change did not reach statistical significance. The γ-secretase activity was more uniformly reduced after all fish oil based diets, although only FO ($p<0.001$) and FOPS ($p<0.05$) groups were significantly different from APdE9 control group. Next, it was addressed whether reduced β- and γ-secretase activities translate into decreased Aβ production in the brain of APdE9 mice after fish oil-based diets. The analysis revealed a significant overall diet effect on the soluble $A\beta_{42}$ ($p<0.00001$), but not on soluble $A\beta_{40}$ levels. However, a closer examination showed a significant decrease in soluble $A\beta_{42}$ ($p<0.001$) levels as well as $A\beta_{42}/A\beta_{40}$ ($p<0.05$) ratio only after FOPS diet. Moreover, no significant diet effects were detected on the levels insoluble $A\beta_{40}$, $A\beta_{42}$, the $A\beta_{42}/A\beta_{40}$ ratio, or on the β-amyloid plaque load in the brain of APdE9 mice. These results imply that diet-associated behavioral phenotypes observed in this study are not explained by alterations in brain β-amyloid pathology.

Neuroinflammation and oxidative stress are two key mechanisms suggested to be involved in the pathogenesis of AD (Heneka et al., 2015). Oxidative damage accompanies the β-amyloid pathology in the brain of AD mouse models (Abdul et al., 2008, Du et al., 2010, Pratico et al., 2001). Plant sterols and some other dietary supplements, such as several vitamins, have been implicated to possess antioxidative properties (Malar and Devi, 2014). Therefore, the effect of fish oil based diets on neuroinflammation and oxidative stress was next assessed in the brain of APdE9 mice. IHC analysis showed that only FOPS diet significantly reduced the overall amount of activated microglia and in the brain of APdE9 mice ($p<0.05$). Other experimental diets did not affect overall or the plaque-surrounding CD45 immunopositive area. In contrast, mice on the FOPS diet were also the only group, which showed significantly increased production of reactive oxygen species (ROS, $p<0.05$) and lysophosphatidylcholine (LPC 16:0 and LPC 18:1; $p<0.05$), a phospholipid with proinflammatory properties (Sundaram et al., 2012), in the brain. Other diets did not affect the levels of ROS or LPCs. Based on these findings, the diet-induced improvements in spatial and olfactory memory are not likely mediated through alleviated neuroinflammation or oxidative stress in these mice. However, in general, in APdE9 mice, higher ROS levels in the cerebellum and hippocampus were associated with a poorer outcome in behavioral tasks, such as reduced rearing ($r=-0.39$, $p<0.01$), increased thigmotaxis (tendency to swim near the pool wall, $r=0.29$, $p<0.05$) and impaired search bias ($r=-0.30$, $p<0.05$). Thus, oxidative stress may be associated with at least some of the behavioral and memory deficits found in APdE9 mice, although these may not necessarily be mediated by dietary factors. Finally, dietary manipulation did not affect the total cortical SFA levels. In contrast, all fish oil based diets led to a consistent increase in the brain *n-3*/*n-6* PUFA ratio (significant in FO group, $p<0.05$), suggesting that the *n3*/*n6* PUFA ratio is modified by dietary supplementation with fish oil.

6 Discussion

6.1 COMPLEX ROLE OF UBIQUILIN-1 IN ALZHEIMER'S DISEASE PATHOGENESIS: STUDIES I AND II

AD is the most common cause of dementia in the elderly, and is soon expected to reach epidemic proportions. Currently, there are no drugs or treatments available for AD that could prevent or halt the disease progression. Furthermore, traditional therapeutic approaches have recently ended up with inconclusive or adverse results in phase III clinical trials (Kumar et al., 2015). Accumulating evidence indicates that deficits in protein trafficking, protein quality control and clearance systems (collectively called proteostasis) in combination with disease-associated genetic and environmental alterations may play a pivotal role in protein aggregation and subsequent neurodegeneration due to their improper clearance (Haapasalo et al., 2010) (Ross and Poirier, 2004) (Wang et al., 2014). Therefore, careful characterization of pathways involved in trafficking, processing and degradation of AD-related proteins is of great importance. As reviewed in study I, ubiquilin-1 has been consistently found to associate with AD pathogenesis at both the genetic and functional points of view, and to specifically regulate trafficking, the levels, and accumulation of several AD-associated proteins (El Ayadi et al., 2012a, Hiltunen et al., 2006, Kleijnen et al., 2003, Liu et al., 2014, Lu et al., 2009, Mah et al., 2000, Massey et al., 2004, Stieren et al., 2011, Viswanathan et al., 2011). However, the data regarding ubiquilin-1 in the brain of humans and other vertebrate animals is scarce and the contribution of ubiquilin-1 to the development and progression of AD pathology still largely remains an open question. In study II, the goal was to investigate the relationship between ubiquilin-1 and key pathological aspects of AD in human brain and in experimental *in vitro* and *in vivo* AD models overexpressing ubiquilin-1. In this study, ubiquilin-1 mRNA and protein levels were found both to be decreased, in relation to advancing AD-related neurofibrillary pathology, in the *post mortem* human temporal cortex. This finding is in line with a previous study showing that ubiquilin-1 protein levels are significantly decreased in late onset AD patient brains ((Stieren et al., 2011)). When ubiquilin-1 protein levels were correlated with other central AD-associated proteins, a trend towards positive correlation between ubiquilin-1 and BACE1 protein levels was observed in human brain. Although this piece of data is partially inconsistent with previous reports demonstrating that BACE1 protein levels are increased in human AD brains (Cole and Vassar, 2007), it suggests that there might exist an interrelationship between ubiquilin-1 and BACE1 levels.

The suggested interrelationship between BACE1 and ubiquilin-1 was supported by the findings in mouse embryonic primary cortical neuron-microglial co-cultures and APdE9 mouse hippocampus *in vivo*. In co-cultures, overexpression of ubiquilin-1 resulted in significantly increased levels of endogenously expressed BACE1, whereas in the mouse brain samples the increase in BACE1 protein levels and activity upon ubiquilin-1 overexpression was highly consistent, but more modest and not statistically significant. However, the milder *in vivo* effect could arise from the fact that the infection efficacy of lentivirus is much higher in cells (~60% neurons were infected) than *in vivo* in mouse brain (~20% of the neurons of the dentate gyrus of hippocampus were infected). Furthermore, a relatively small group size

(n=7-8 mice/group) in the *in vivo* experiment, may partially explain the lack of significance in this study. Experiments in different cultured neuronal cells using co-overexpression of ubiquilin-1 and different BACE1 constructs (with or without the endogenous 5′ and 3′ UTRs) further corroborated this increase in BACE1 levels. In these cells, stable or transient overexpression of ubiquilin-1 significantly increased the protein levels of all three BACE1 constructs and this increase was reversed by ubiquilin-1 downregulation by RNAi. A similar increase was not observed in endogenously expressed ERK1/2 or overexpressed GFP, suggesting that human ubiquilin-1 might specifically interact with BACE1. Finally, the levels of ubiquilin-1 and BACE1 were significantly correlated in these cells, suggesting a strong association between these proteins. Altogether, these data indicate an interrelationship between ubiquilin-1 and BACE1 in the human brain, *in vivo* in the mouse brain and *in vitro* in primary neurons and cultured neuronal cells.

The increase in BACE1 protein levels in ubiquilin-1-overexpressing cells may involve several alternative underlying molecular mechanisms. Increased BACE1 levels upon ubiquilin-1 overexpression in cells transfected with the BACE1-myc construct, which contains the very potent CMV-promoter (Choi et al., 2005) but lacks the endogenous UTRs, suggests that ubiquilin-1 increases BACE1 levels by a post-translational mechanism(s). The following studies indicated that overexpression of ubiquilin-1 significantly increased the half-life of BACE1 protein, suggesting that BACE1 levels are stabilized in cells overexpressing ubiquilin-1. At the same time, BACE1 localization in Rab7-positive LELs was decreased, while it was modestly increased in TfR-positive early endosomal compartments in cells overexpressing ubiquilin-1. Although there was no evidence for altered levels of GGA1, GGA3, ARF6, seladin-1, or BACE1 ubiquitination, factors known to regulate BACE1 trafficking (Kang et al., 2010, Kang et al., 2012, Sannerud et al., 2011, Sarajarvi et al., 2009, Tesco et al., 2007), it is possible to suggest that less BACE1 is targeted to the lysosomal degradation pathway in cells overexpressing ubiquilin-1. As has been reviewed in study I, this finding agrees with the previously indicated role for ubiquilin-1 in the regulation of intracellular trafficking of several different proteins (Hiltunen et al., 2006, Lu et al., 2009, Massey et al., 2004, Thomas et al., 2006, Viswanathan et al., 2011, Viswanathan et al., 2013). Collectively, these data suggest that ubiquilin-1 affects BACE1 levels at the post-translational level and stabilizes BACE1 by diverting it from the lysosomal degradation pathway. However, unraveling the exact molecular mechanisms by which ubiquilin-1 mediates BACE1 subcellular trafficking still requires further investigations.

BACE1 is a rate-limiting enzyme in Aβ production (Cole and Vassar, 2007). Therefore, the finding that ubiquilin-1 overexpression increases BACE1 levels is partially in contrast with previous studies showing that downregulation of ubiquilin-1 increases APP processing and Aβ production (El Ayadi et al., 2012a, Hiltunen et al., 2006, Stieren et al., 2011). On the other hand, another study conducted in a different cell line has suggested an opposite effect on APP by ubiquilin-1 knockdown (Zhang et al., 2007). Based on these studies, it is possible that ubiquilin-1-mediated regulation of APP is cell type-specific. In study II, the effects of ubiquilin-1 on APP processing and Aβ pathology have been investigated for the first time *in vivo* in the brain of APdE9 transgenic AD mice. In this study, there were no significant changes in APP ubiquitination, maturation, levels of APP metabolites or plaque load associated with ubiquilin-1 overexpression in the brain of APdE9 mice. However, a trend

towards an increase in the levels of soluble $A\beta_{40}$ and $A\beta_{42}$ as well as insoluble $A\beta_{40}$ were found in the hippocampi of lentiviral expressing ubiquilin-1-injected mice as compared to controls. At the same time, Aβ plaque load was contrastingly found to be moderately decreased in the brain upon ubiquilin-1 overexpression. This inconsistency between plaque load and Aβ levels could be due to the fact that the Aβ molecules continuously aggregate into and dissolve from the plaques, which have been suggested to be dynamic structures (Maggio et al., 1992). Based on this, it is possible that overexpression of ubiquilin-1 could somehow modulate the balance between aggregated and soluble forms of Aβ. All in all, together with previous *in vitro* data reviewed in detail in study I, it appears plausible that ubiquilin-1 influences Aβ pathology at multiple levels, by regulating both APP and BACE1 and by possibly the flux state of Aβ aggregation. A recent, and currently the only study, which has previously elucidated the role of ubiquilin-1 in mouse brain, found that knockdown of ubiquilin-1 leads to the accumulation of ubiquitinated proteins and an aggravation of neuronal damage, while lifelong ubiquilin-1 overexpression protects the mice from neuronal insults and the prolonged motor defects that occur after ischemic insult or oxidative stress (Liu et al., 2014). Thus, it is possible that, if induced earlier, the effects of ubiquilin-1 overexpression on Aβ pathology might be stronger than those observed in the present study. In any follow up studies, it would also be interesting to determine how ubiquilin-1 knockdown would influence APP processing or Aβ pathology in the brain of APP transgenic mice under excessive AD-associated stress.

Accumulating evidence indicates that neuroinflammation plays a role in the AD pathogenesis (Heneka et al., 2015). Ubiquilin-1, on the other hand, has been suggested to alleviate different stress effects *in vitro* and *in vivo,* but its role in neuroinflammation had not been characterized previously ((Ko et al., 2002, Lim et al., 2009, Liu et al., 2014, Lu et al., 2009)). In study II, ubiquilin-1 overexpression was not observed to significantly influence the inflammatory response during LPS + IFN-γ –induced neuroinflammation in co-cultures of mouse embryonic primary cortical neurons and BV2 microglial cells. Conversely, overexpressed ubiquilin-1 was upregulated by the LPS + IFN-γ treatment, suggesting that ubiquilin-1 somehow responds to neuroinflammatory stress. These results altogether suggest that under this type of acute inflammatory stress, the up-regulation of ubiquilin-1 levels does not lead to an alleviation of neuroinflammation in a similar manner to the case of ER stress (Ko et al., 2002, Lu et al., 2009), but instead it may aggravate the subsequent neurodegeneration. There were no ubiquilin-1-associated alterations in the activity of Akt, which is a kinase mediating neuronal survival via activation of a prosurvival signaling cascade (Ghosh and Greenberg, 1995). Therefore, the mechanism of compromised neuronal survival upon ubiquilin-1 overexpression will remain a matter of investigation in future studies. All in all, together with the previous data, these findings suggest that ubiquilin-1 may have divergent roles depending on the prevailing stress conditions. According to previous data showing that BACE1 expression is increased by both LPS and IFN-γ-induced inflammation, here BACE1 levels were also modestly, although not significantly, upregulated under inflammation.

The final aim of the study II was to investigate the previously uncharacterized relationship of ubiquilin-1 and tau *in vitro* and *in vivo*. Ubiquilin-1 has been shown to be detectable in NFTs, which primarily contain aggregated, hyperphosphorylated tau in AD

brain, suggesting that there may exist a relationship between ubiquilin-1 and tau (Mah et al., 2000, Mizukami et al., 2014). In cultured non-neuronal cells, ubiquilin-1 overexpression led to a significant increase in the levels of the predominant adult 0N4R-tau protein isoform and its phosphorylation at the AD-specific AT8 epitopes. However, a similar effect on tau was not observed in co-cultures of mouse embryonic primary cortical neurons and BV2 microglial cells, although ubiquilin-1 slightly augmented LPS + IFN-γ-induced increase in total 0N3R-tau protein levels. As the 0N3R-isoform is the predominant form of tau expressed in neurons during embryogenesis and possess a completely different role than adult tau (Lee et al., 2001), it is plausible that ubiquilin-1 differentially regulates divergent tau isoforms during different developmental stages. In contrast to this prediction, no major alterations were detected in endogenously expressed 0N4R-tau isoform levels or phosphorylation *in vivo* in the hippocampus of APdE9 mice after ubiquilin-1 overexpression. However, it should be noted that APdE9 mice do not develop tau aggregates, and ubiquilin-1 has been shown to associate especially with NFTs in human brain (Mah et al., 2000, Mizukami et al., 2014). Probably, it would be more relevant to elucidate the interaction between tau and ubiquilin-1 in mice overexpressing human tau. Future studies will allow a better understanding of the potential interaction of ubiquilin-1 and tau and the underlying molecular mechanisms leading to increased tau levels under cellular stress.

6.2 THE EFFECT OF DIETARY LIPID COMPOSITION ON ALZHEIMER'S DISEASE-RELATED PATHOGENESIS AND COGNITIVE DECLINE: STUDIES III AND IV

6.2.1 High-fat diet increases tau expression and exon 10 inclusion in the brain of female mice independently of genotype, peripheral or central insulin signaling or neuroinflammation status: Study III

Due to the anticipated increase in the numbers of people suffering from AD and the fact that there is currently no cure for this affliction, it is of great importance to identify efficient strategies for reducing the risk of developing or preventing the progression of this disease. Accumulating evidence indicates that one plausible approach to intervene with disease development might be dietary manipulation. AD by itself as well as several AD risk factors, including obesity and T2DM, are directly associated with dietary choices (Morris and Tangney, 2014, Sindi et al., 2015). HFD, which contains considerable amount of SFAs, is a common way to induce obesity and/or T2DM in rodents and several lines of evidence suggest that HFD accelerates cognitive decline and AD-related pathology in mouse models of AD (Ho et al., 2004, Julien et al., 2010, Kohjima et al., 2010, Oksman et al., 2006, Takeda et al., 2010, Vandal et al., 2014). In particular, several recent studies have demonstrated that a HFD and high calorie surplus over energetic expenditure induce the expression and/or phosphorylation of tau in mice, although the functional significance of these observations have remained unclear (Bhat and Thirumangalakudi, 2013, Jeon et al., 2012, Koga et al., 2014, Leboucher et al., 2013, Moroz et al., 2008). In the study III, HFD was found to elicit a significant increase in the protein levels, mRNA expression and exon 10 splicing of endogenous murine tau in the brain of female mice, regardless of the AwIw (wild-type), AwI+ (IGF2), A+Iw (APdE9) or A+I+ (APdE9xIGF2) genotype. This finding coincided with the previously reported upregulation of phosphorylation of tau in the protein lysates

extracted from the temporal cortex of these mice after HFD (Hiltunen et al., 2012). In contrast, male mice on HFD did not show similar increase in the expression, splicing or phosphorylation of tau. Related to this, it has previously been shown that the Aβ pathology in APdE9 mice progress faster in females compared to males (Wang et al., 2003) and that dietary supplementation with DHA protects female, but not male, mice from amyloid pathology (Perez et al., 2010). The reason of this difference between the genders remains thus far unclear and further studies are required for instance in ovariectomized mice combined with dietary manipulation. Importantly, the diet-induced increase in tau mRNA expression in female mice was correlated with poorer performance in spatial memory test and reduced explorative activity in the novel environment. However, as the most significant impairment of spatial memory was strongly associated with A+I+ genotype (Hiltunen et al., 2012), it seems plausible that increased tau expression contributes to, but is insufficient on its own, to cause severe cognitive impairment (Hiltunen et al., 2012). Taken together, it is likely that in addition to diet, gender-specific factors contribute to the tau pathology and cognitive decline.

Although 3R-tau levels are generally low in the brain of adult mice, a recent study demonstrated that 3R-tau is abundantly expressed at least in certain brain areas of three-month-old wild-type mice (McMillan et al., 2008). In the present study III, 3R-tau protein levels were below the detection level in the temporal cortex of aged female mice. However, optimization of the PCR conditions separately for 3R- and 4R-tau allowed the quantification of mRNA levels of 3R-tau and to addressing the effects of genotype and diet on the 3R-tau expression and alternative splicing of exon 10. Although the pathogenic role of aberrant tau splicing is a matter of debate in AD, it has been demonstrated that increased levels of 3R tau may play a role in the progression of tau pathology during AD (Connell et al., 2005, Espinoza et al., 2008, Glatz et al., 2006, Ingelsson et al., 2006). In contrast, most of the familial tau mutations associated with neurodegenerative tauopathies lead to the imbalanced splicing of the tau exon 10, so that the 4R-tau isoforms become over-represented (Lee et al., 2001). In the current study, a significant diet-induced increase in 4R-tau to 3R-tau mRNA ratio was detected in the brain of female mice regardless of the genotype. This suggests that HFD does not only promote tau expression, but may also alter tau exon 10 alternative splicing. Recently, it was demonstrated that the expression levels of tau splicing factors, such as Tra2β, may be altered during obesity and abnormal peripheral insulin signaling in humans and animal models (Jung et al., 2011, Pihlajamaki et al., 2011). Therefore, the involvement of expressional changes in the tau exon 10 splicing enhancers was elucidated in the brain of HFD mice. Even though HFD significantly increased the ratio of 4R-tau to 3R-tau in this study, there were no diet-related effects on the expression of tau exon 10 splicing enhancers, Traβ, SC35, SRp30c and SF2/ASF (Liu and Gong, 2008, Qian and Liu, 2014). This does not, however, rule out the possibility that the expression of tau exon 10 splicing silencers were affected by the HFD. Furthermore, it should be noted that the activity of tau splicing factors are primarily regulated through changes in their phosphorylation status, which was not assessed here (Hartmann et al., 2001, Qian and Liu, 2014). In contrast to the 4R-tau expression, which was exclusively influenced by the diet, the mRNA levels of 3R-tau were also significantly altered by the genotype. More precisely, the mRNA levels of 3R-tau were increased in A+I+ mice on standard diet as compared to A+Iw or AwI+ mice, and this was not further augmented by the HFD. Since the accelerated T2DM phenotype was observed only in the A+I+ mice

(Hiltunen et al., 2012), it is thus likely that the expression and/or splicing of individual tau isoforms are mechanistically differentially regulated by the diabetic phenotype and the HFD. This idea agrees with the recent study, which similarly reported a specific increase in the levels of 3R-tau, accompanied by the increased expression of exon 10 splicing silencer, Srp55c, in the rat model of T2DM (Jung et al., 2011). Notably, another study recently demonstrated that Aβ may modulate tau exon 10 splicing via activating GSK3β, which in turn phosphorylates exon 10 splicing factor, SC35, and thus reduces the relative amount of 4R-tau (Chen et al., 2010). It is interesting to note, that the expression of SC35, Srp30c and SF2/ASF were slightly, although not significantly increased in A+, but not in Aw mice after HFD in the current study. Since a similar trend of modest reduction in the inhibitory phosphorylation of GSK3β was detected in A+ mice after HFD, it is plausible that increased Aβ levels may contribute to increased 3R-tau expression in A+ mice. However, this idea needs to be investigated in the future studies by determining the phosphorylation of SC35, Srp33c, and SF2/ASF. All in all, these findings suggest that the delicate balance between the expression of different tau isoforms is compromised by the HFD regime, although further investigations are needed to comprehensively assess the exact underlying mechanisms of this derangement.

It has been suggested that diet-induced obesity and related dyslipidemia, abnormal peripheral and/or central insulin signaling may underlie tau-related changes in mice (Bhat and Thirumangalakudi, 2013, Jeon et al., 2012, Koga et al., 2014, Leboucher et al., 2013, Moroz et al., 2008). Thus, association between tau levels and weight gain, peripheral glucose metabolism, and brain insulin signaling was elucidated order to address whether these factors influence the diet-related tau changes. Interestingly, increased phosphorylation of tau has been demonstrated in the brain of cognitively intact morbidly obese individuals, suggesting that obesity may in fact predispose animals to tau-related changes (Mrak, 2009). In the current study, the weight gain observed in the female HFD mice positively correlated with 4R-tau and 3R-tau expression as well as increased 4R-tau to 3R-tau ratio. However, since the weight gain in the female A+Iw mice on the HFD did not reach statistical significance, it cannot exclusively explain the tau-related changes. Furthermore, neither 4R-tau, nor 3R-tau expression, nor exon 10 splicing were associated with peripheral changes in glucose metabolism. This agrees with the previous study, where sustained hyperglycemia as well as insulin resistance and hyperinsulinemia were observed only in the A+I+ mice (Hiltunen et al., 2012). The relationship between central insulin signaling and tau-related pathology after HFD have yielded conflicting results in animals (Becker et al., 2012, Jeon et al., 2012, Leboucher et al., 2013, Moroz et al., 2008). It is well-established that the Akt/GSK3β signaling pathway has a pivotal role in the central insulin resistance and molecular mechanisms of AD (Jope and Johnson, 2004). Here, the HFD did not affect the activity of Akt or GSK3β in the temporal cortex, implying that diet-related tau changes are not mediated by impaired central insulin signaling via Akt/GSK3β signaling pathway. The findings regarding the association between peripheral and central insulin signaling and diet-induced tau changes are in accordance with a recent study, which reported that HFD-induced obesity worsens tau pathology independently of peripheral or central insulin resistance in THY-tau22 transgenic and wild-type mice (Leboucher et al., 2013).

Finally, neuroinflammation is a potential mechanism that could link neurodegenerative diseases, T2DM (Jope and Johnson, 2004) and tau-related changes during diet-induced obesity (Julien et al., 2010, Koga et al., 2014, Moroz et al., 2008). Based on this idea, the effects of HFD on astrogliosis and the expression of proinflammatory cytokines, TNFα and IL1β, were investigated. As was previously shown, HFD exposure does not affect microglial activation in these mice (Hiltunen et al., 2012). Consistent with that, there were no diet-related effects on astrogliosis in the brain of female mice. These findings are partially in contrast with other studies, where HFD and genetically-induced diabetes promoted cerebral inflammation in mice (Jeon et al., 2012, Julien et al., 2010, Koga et al., 2014, Moroz et al., 2008, Takeda et al., 2010). On the other hand, the mRNA, but not protein levels of TNFα were significantly decreased after HFD and negatively associated with tau-related changes, suggesting that diet-induced expression and splicing of exon 10 of tau are not mediated by exacerbated neuroinflammation. However, given that TNFα is a protein that is secreted from the cells into the interstitial space, it is plausible that the mRNA levels of TNFα may in fact reflect local inflammation status better than the protein levels in the temporal cortex. Thus, it cannot conclusively ruled out that some other parts of the brain outside of the temporal cortex could show aggravated cerebral inflammation due to the HFD.

Altogether, the present data in conjunction with previous results from these mice lend support to the notion that the changes in tau expression and tau exon 10 inclusion are influenced by dietary lipids and altered lipid profile rather than diabetes-related changes (both central and peripheral) or neuroinflammation. In line with this hypothesis, it was recently reported that certain lipids may play a role in triggering neuroinflammation and neurodegeneration, suggesting that diet-induced dyslipidemia may influence cerebral conditions (Sundaram et al., 2012). In addition, previous reports indicate that cholesterol dyshomeostasis increase tau phosphorylation and cholesterol-lowering compounds are protective in tau transgenic animals (Boimel et al., 2009, Glockner et al., 2011) (Grimm et al., 2013). Furthermore, accumulating evidence from experimental studies suggest that the levels of DHA and plan sterols directly modulate AD-type pathology in the brain (Calon et al., 2005, Grimm et al., 2011, Julien et al., 2010, Morris et al., 2003b, Oksman et al., 2006). However, it remains to be determined in future studies whether these mice suffer from dyslipidemia after HFD.

Another plausible mechanism for possible diet-induced tau pathology, which was not addressed here, is oxidative stress. Oxidative stress is thought to be centrally involved in the pathogenesis of both AD and T2DM (Rosales-Corral et al., 2015) and has been shown to precede amyloid pathology in the brain of AD mouse models (Abdul et al., 2008, Du et al., 2010, Pratico et al., 2001). In support of this idea, a recent study reported that long-term HFD consumption leads to hippocampal-dependent memory defects, which were associated with mitochondrial dysfunction in the transgenic mouse model of AD (Petrov et al., 2015). On the other hand, mitochondrial dysfunction and oxidative stress were directly linked to tau-related pathology *in situ* in human hippocampal neurons during AD and *in vitro* model of tau pathology in another recent study (Spilsbury et al., 2015). Therefore it remains to be addressed in future studies whether HFD and tau-related changes are connected through mitochondrial dysfunction or oxidative damage.

6.2.2 Additional dietary supplements modulate beneficial effects of fish oil based diets: Study IV

The data from epidemiologic and experimental studies suggest that dietary supplementation with a *n-3* PUFA, DHA, may decrease AD risk and protect from AD-related pathological changes, whereas DHA depletion and increased *n-6*/*n-3* ratio may have the opposite effect (Calon et al., 2004, Calon et al., 2005, Grimm et al., 2011, Julien et al., 2010, Laitinen et al., 2006, Ma et al., 2009, Morris et al., 2003b, Oksman et al., 2006, Teng et al., 2015). Despite the evident beneficial effect of fish oil and other *n-3* PUFA sources in these studies, it has been suggested that the protective effects cannot be attributed to individual nutrients and that DHA alone might be insufficient in providing protection from AD-related pathological changes (Lim et al., 2006). Presumably, the combined intake of selected nutritional components, such as plant sterols or UMP, choline, phospholipids, folic acid, vitamins B6, B12, C, E, and selenium, which are precursors and cofactors for synaptic membrane synthesis, may be required in order to reach true efficacy (Broersen et al., 2013, Cansev et al., 2015, de Waal et al., 2014, de Wilde et al., 2011, Jansen et al., 2013, Jansen et al., 2014, Malar and Devi, 2014, Scheltens et al., 2010, Scheltens et al., 2012, van Wijk et al., 2014). Study IV demonstrates that the effects of fish oil based diet on cognition and amyloid pathology can be modified by additional nutrients in the APdE9, AD, mouse model. Whereas all fish oil-containing experimental diets (FO, FOPS and Fortasyn) were found to fully restore the impaired olfactory recognition in APdE9 mice, only additional specific multinutrient supplementation found in Fortasyn alleviated their deficit in spatial memory. This is consistent with the previous studies, where DHA-containing diets were reported to improve the performance of AD mice in object recognition tests (Arsenault et al., 2011, Kariv-Inbal et al., 2012), but not in Morris swim navigation task measuring spatial memory and learning (Arendash et al., 2007, Oksman et al., 2006). Divergent behavioral benefits of different dietary supplements might arise from the fact that memory tasks used in the study require the function of different brain circuits and different plasticity mechanisms. In other words, it has been shown that recognition memory is mainly dependent on long-term synaptic depression (Griffiths et al., 2008), while hippocampal dependent spatial memory requires long-term synaptic potentiation (Morris et al., 2003b). Cholinergic signaling, on the other hand, may be central for function of both memory types (Cansev et al., 2015, Miranda et al., 2009, Warburton et al., 2003). Agreeing with the idea that Fortasyn may specifically modulate memory functions associated with cholinergic neurotransmission, a recent study reported that this multinutrient composition efficiently enhanced hippocampal cholinergic signaling in aged rats (Cansev et al., 2015). In addition to this finding, mice receiving the FOPS diet were found to be the poorest learners in the Morris swim task but showed the fastest habituation to the novel test cage in the current study. Although both are related to synaptic spatial memory, it is possible that the confounding results are influenced by significantly increased body weight observed in the FOPS group. Besides memory defects, anxiety, restlessness and depression are common features in AD (Ferretti et al., 2001). Previous experimental studies have suggested that dietary supplementation with the Fortasyn, but not fish oil alone, may alleviate anxiety-related behavior in AD mouse models (Jansen et al., 2013, Jansen et al., 2014). In contrast to these observations, none of the experimental diets in the current study were able to reduce the anxiety-related hyperactivity or increase the reduced

exploratory behavior in APdE9 mice. However, it should be noted that the test settings, viz. the monitoring time and the size of the test cage, were markedly different in the previous studies as compared to the ones used in the current study. Thus, it is plausible that anxiety-related dietary effects were not as evident with the shorter monitoring periods used in the current study. All in all, these data suggest that additional nutrients included in the Fortasyn may potentiate the beneficial cognitive effects of a fish oil-based diet.

Previous studies have shown that DHA supplementation reduces Aβ burden by several pleiotropic mechanisms, such as inhibiting β- and γ-secretase activity (Grimm et al., 2011, Oksman et al., 2006, Zhao et al., 2011). Consistent with these notions, both β- and γ-secretase were significantly inhibited in the brain of aged APdE9 mice after 9 months of dietary intervention with FO diet. However, in the current study the secretase activities were further influenced by added nutrients, so that the β-secretase activity was not markedly affected by the FOPS diet, whereas γ-secretase activity was more uniformly reduced by all experimental diets. In spite of evident secretase inhibition, none of the diets affected the amount of amyloid plaques or the levels of insoluble Aβ, whereas soluble Aβ levels were reduced only in mice on FOPS diet. These results are partially in contrast with previous studies, which have shown that both Fortasyn and DHA alone may alleviate Aβ toxicity and burden in the brain of mouse and rat models of AD (Broersen et al., 2013, de Wilde et al., 2011, Oksman et al., 2006). However, it should be taken into account that in these dietary intervention studies, diet started significantly earlier or the animals were significantly younger at the time of sacrifice. Therefore, it seems plausible that the beneficial effects of dietary manipulation on amyloid pathology strongly depends on the onset age and/or duration of the intervention. Furthermore, the previous reports regarding the effects of dietary DHA on Aβ pathology have not been unequivocal, and it has been suggested that its beneficial effects may depend upon the composition of the diet (Amtul et al., 2011, Arsenault et al., 2011, Jansen et al., 2013). Taken together, as Fortasyn diet was the only diet associated with improved spatial learning and odor recognition, it is evident that alleviation of APdE9 transgene-related cognitive impairment by the dietary intervention was unrelated to changes in Aβ levels. Furthermore, since Fortasyn Connect did not have significant effects on the ROS production, the amount of active microglia or the levels of proinflammatory LPCs, it is highly unlikely that improved cognitive performance was mediated by anti-inflammatory or antioxidative properties of these nutrients.

Finally, based on the current findings, the role of dietary plant sterol supplementation remains inconclusive. Diet-induced γ-secretase inhibition led to a significant decrease in the Aβ42/40 ratio only in the FOPS group, indicating that plant sterol supplementation leads to γ-secretase modulation. However, whereas the FOPS diet significantly reduced brain microglial activation, it was also the only diet that significantly increased hippocampal ROS production and neuroinflammation in terms of increased LPCs. In a recent report these phospholipids were shown to trigger astrogliosis and neurodegeneration in primary neuronal cultures (Sundaram et al., 2012). Therefore, despite a promising action on Aβ accumulation, the plant sterol supplementation may have adverse effects on neuroinflammation and production of reactive oxygen species, which may prevent its otherwise beneficial effects on brain function in the AD model mice.

7 Conclusions

The aim of this thesis was to investigate the effects of specific genetic and dietary factors on AD-related molecular pathology by conducting experiments in well-established *in vitro* and *in vivo* models and analyzing human *post mortem* brain tissue samples. In these studies, the special emphasis was on the function of AD-associated ubiquilin-1 (Studies I and II) and on the influence of dietary nutrient composition (studies III and IV). Following conclusions can be drawn based on the findings in these studies.

I. Together, the findings from human AD brain and from *in vitro* and *in vivo* models in Study II suggest a novel interrelationship between ubiquilin-1 and BACE1, which leads to BACE1 stabilization by diverting it from the lysosomal degradation pathway. This interaction may thereby play a role in the regulation of Aβ accumulation and AD pathogenensis.

II. Data obtained from study II also indicate that ubiquilin-1 does not alleviate the AD-associated neuroinflammation in a similar manner as it has been shown to alleviate other stress conditions in *in vitro* models. Thus ubiquilin-1 may play a divergent roles during different stress conditions.

III. As a summary of Study I and novel data obtained from Study II, ubiquilin-1 might represent a common mechanistic link between different neurodegenerative diseases. However, the data regarding the effects of ubiquilin-1 in AD and neurodegeneration is still far from complete. At present, it is quite evident that due to its complex interactome and functions, ubiquilin-1 would be a complicated therapeutic target in AD. Future studies will allow better understanding of the effects of ubiquilin-1 modulation on AD-related pathological pathways and whether it possesses translational potential.

IV. The data from Study III suggest that a HFD, rich in SFAs, increases tau expression and exon 10 inclusion and this may lead to cognitive decline in mice. The data also suggest that the expression of 3R-tau and 4R-tau isoforms may be regulated through different mechanisms. As a conclusion, HFD might associate with increased risk or faster progression of AD and other neurodegenerative tauopathies.

V. Study IV confirmed that fish oil has beneficial effects in a mouse model of AD. Additional supplementation, with other nutrients supporting neuronal membrane formation , may enhance the cognitive benefits of fish oil by improving synaptic plasticity through Aβ-independent mechanisms. On the other hand, plant sterols may potentiate the Aβ-lowering effect of DHA/fish oil, but more research is needed to find a suitable formulation for their long-term use with the aim of AD prevention.

VI. Although the role of dietary lipids or other dietary supplements in AD still remains a matter of investigation, together the findings from Studies III and IV indicate that dietary lipids may play a role in the regulation of AD-related pathological mechanisms and cognitive decline. Thus, dietary modulation may be an effective way to influence AD risk or progression at a global level.

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Mari Takalo

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Several genetic and lifestyle factors influence the risk for Alzheimer's disease (AD), a complex neurodegenerative disorder. This thesis focuses on the characterization of the effects of *UBQLN1* gene and dietary lipids on the molecular mechanisms of AD in preclinical models and human brain. These findings advance our knowledge of AD molecular pathogenesis and may be applied in the development of novel therapeutic approaches and intervention strategies in the future.

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